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(54) Title: METHOD AND KIT FOR DETECTING GENOMIC INSTABILITY

(57) Abstract

A method of determining genomic instability is disclosed. The method includes the steps of isolating a cell sample and isolating DNA from the cell sample. The isolated DNA is then analyzed by neutral-neutral 2D gel electrophoresis and from the electrophoresis arc pattern the presence of heterogenous circular DNA molecules is determined. The presence of circular DNA indicates genomic instability.

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METHOD AND KIT FOR DETECTING GENOMIC INSTABILITY

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to a method of determining genetic instability in cells and more specifically a method for screening for carcinogens and for identifying pre-malignant cells.

BACKGROUND ART

Genomic instability is a hallmark of tumor cells [Fidler and Hart, 1982; Nowell, 1976] and can be enhanced by carcinogen treatment [Brown et al., 1983; Eki et al., 1987; Goz et al., 1989; Hahn et al., 1986; Kleinberger et al., Morgan et al., 1986; Rice et al., 1987; Tlsty et al., 1989]. This phenomenon consists of translocations, aneuploidy, recombinations, deletions, gene amplification and the appearance of small polydisperse circular (spc) DNA. Gene amplification is the most studied process of genomic instability [for reviews see references Schimke, 1988 and Windle and Wahl, 1992]. It is a dynamic process and the amplified sequences are either associated with the chromosome, as expanded chromosomal regions, or they can be extrachromosomal and represented as acentric circular structures known as double minute chromosomes.

Structural analysis showed that the amplified sequences are often organized as chromosomal or extrachromosomal inverted repeats (IRs). The possible involvement of inverted duplications in the generation of amplified DNA was suggested [Ford et al., 1985; Ford and Fried, 1986; Fried et al., 1991, Heartlein and Latt, 1989; Hyrein et al., 1988; Looney and Hamlin, 1987; Ma et al., 1988; Nalbantoglu and Meuth, 1986; Passananti et al., 1987; Ruiz and Wahl, 1988; Ruiz and Wahl, 1990]. In

some cases the early amplification products were organized as extrachromosomal circles containing IRs. The formation of these circles was proposed to be one of the initial steps of gene amplification [Carroll et al., 1987; Passananti et al., 1987; Ruiz and Wahl, 1988; Wahl, 1989; Windle et al., 1991; Windle and Wahl, 1992;]. Chromosomal breaks, ligations and recombinations could explain the formation of these extrachromosomal circles [Carroll et al., 1987; Carroll et al., 1988; Ruiz and Wahl, 1988; Ruiz and Wahl, 1990; Windle et al., 1991, for reviews see references Wahl, 1989 and Windle and Wahl, 1992]. However the very early steps of the process could not be detected since in all these systems the amplified DNA was analyzed only upon selection, when the cells acquired a distinguishable phenotype, several generations after the initial amplification event.

An additional phenomenon, which appears in normal cells but was shown to increase in correlation with genomic instability, is the formation of small polydispersed circular (spc) DNA (for reviews, see references 24, 70 and 90). spcDNA is found in cell lines from different organisms, such as *Drosophila melanogaster*, mouse, hamster, monkey and human [Assum et al., 1989; Assum et al., 1993; Bertelsen et al., 1982; Flores et al., 1988; Fujimoto et al., 1985; Krolewski et al., 1984; Krysan et al., 1989; Krysan and Calos, 1991; Kunisada and Yamagishi, 1984; Kunisada and Yamagishi, 1987; Misra et al., 1989; Misra et al., 1987; Motejlek et al., 1993; Stanfield and Helinsky, 1976; Stanfield and Helinski, 1984; Sunnerhagen et al., 1989; Sunnerhagen et al., 1986; Yamagishi et al., 1983]. Their sequences represent the whole genome and they are thought to be generated from the chromosomes. Enhanced amounts of spcDNA can be obtained following treatment with drugs arresting DNA replication [Sunnerhagen et al., 1989; Sunnerhagen et al., 1986] and were correlated with genomic instability [Motejlek et al., 1991; Motejlek et

al., 1993; Wahl, 1989]. The mechanism of spcDNA formation is still obscure however, it may be similar to some of the mechanisms of gene amplification and to other phenomena which characterize genomic instability such as recombinations, deletions and translocations.

It would be useful to be able to identify cells in which the earliest events associated with genomic instability are occurring. It would allow identification of cells that were entering a malignant phase and allow for earlier treatment. Further, genomic instability is associated with exposure to carcinogens [Brown et al., 1983; Eki et al., 1987; Goz et al., 1989; Hahn et al., 1986; Kleinberger at al., 1986; Morgan et al., 1986; Rice et al., 1987; Tlsty et al., 1989]. It would be useful in *in vitro* tests to be able to screen for the early occurrence of genomic instability as a method of identifying carcinogens as well as to determine individual susceptibility and exposure.

In general the bioassay of potential carcinogens involves initial *in vitro* short term screening in bacteria (as for example the Ames test) and/or mammalian cell lines followed by short term *in vivo* bioassays in animals and, depending on the short term results, chronic animal testing. However, the tests are not always as predictive as one would like. For example, in the Ames type tests of measuring bacterial mutagenesis, a mammalian enzyme preparation is required to provide for metabolism of procarcinogens. The enzyme preparation is the limiting part of the test. United States Patent 4,256,832 provides a carcinogenic screening assay in which provides a more rapid assay than that of the Ames test and which is based on oxygen consumption of the test organism. The test system is set up to measure reversion to a wild type form of the test organism. However, while a rapid test it is limited to cell types that have a mutant form which can revert to the wild type and which are capable of rapid growth in only one of its mutant or

wild type form. In screening for carcinogens it would be useful to be able to screen normal human cells for their response to the carcinogen, rather than special cell types or bacteria. It would be useful to have additional methods of measuring potential carcinogens and in particular to be able to use genomic instability to identify carcinogens. Further, given the cost of these procedures both *in vitro* and *in vivo*, it would be useful to have additional *in vitro* methods for screening that require less time and are more predictive of carcinogenic activity.

Genetic rearrangements can be a part of the cellular differentiation pathways and may play a role in developmental and aging processes. It has been proposed that spcDNA is the by-products of genetic rearrangements such as deletions and applications. Furthermore, chromosomal sequences have been reported to be unstable during aging of cells from higher organisms. This indicates that spcDNA formation is correlated with various manifestations of mammalian aging processes. Several studies (Kunisada et al. 1985; Yamagishi et al., 1985) have demonstrated that aged cells were characterized by genomic instability and in such cells a significant increase in spcDNA was detected both *in vivo* (in cells taken from old donors) and *in vitro* (in cells grown in culture for many generations). High levels of spcDNA were found in cells taken from patients suffering from Werner's syndrome which manifest acceleration of many aged-associated disorders (Kunisada et al., 1985). Moreover, the FA cells, which contain high levels of spcDNA (Motejlek et al., 1993; Example 7 herein below), were reported to display early aging *in vitro* and their replicative life span was greatly diminished compared to that of normal cells (Thompson and Holliday, 1983; Schindler and Hoehn, 1988). It would be useful to be able to monitor for extrachromosomal circular DNA and particularly spcDNA *in vivo* and *in vitro* for studies of

age related disorders, particularly for disorders manifesting themselves earlier than would be expected in a normally aging mammal.

As noted herein above, extrachromosomal circular DNA and particularly spcDNA is one of the earliest indications of genomic instability. However, again as noted above, the early identification of the circular DNA could not be detected since in all the studied systems the amplified DNA was analyzed only upon selection, when the cells acquired a distinguishable phenotype, several generations after the initial amplification event. Applicants therefore searched for an easy and convenient approach to detect and to analyze such molecules at their earliest occurrence. The separation of circular DNA on the CsCl-EtBr density gradient is commonly used. However, it requires the preparation of huge amounts of extrachromosomal DNA and is inefficient due to the loss of a significant fraction of the circular molecules - the relaxed circles - which migrate with the linear DNA. Moreover, this approach does not facilitate further characterization of the circular molecules other than by cloning or electron microscopy.

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, a method of determining genomic instability is disclosed. The method includes the steps of isolating a cell sample and isolating intact genomic DNA from the cell sample. The isolated DNA is then analyzed by neutral-neutral (buffer pH) 2D gel electrophoresis and the presence of heterogeneous, i.e. incrementally sized, circular DNA molecules is determined from the presence of the electrophoresis arc pattern. The presence of the reproducible arc pattern is a positive indication of heterogeneous incrementally sized circular DNA molecules which indicates genomic instability.

The neutral-neutral 2D gel analysis of the present invention serves as a very useful tool for the detection, isolation and analysis of extrachromosomal circular DNA, as for example spcDNA, in various cell lines, in normal and tumor cells under different growth conditions and in patient cell samples. This technique facilitates the direct characterization of the DNA included in the circular population and the detection of the sequences that are enriched as well as being an early indicator of genomic instability.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a diagram of the "U turn" replication model and show formation of hairpin structures at arrested replication forks and their conversion into circular inverted dimers [adapted from reference Cohen et al., 1994];

FIGURE 2A-D are photographs of a neutral-neutral 2D gel analysis of defective SV40 molecules showing an arc pattern similar to that of extrachromosomal MNNG-treated CO60 DNA, defective SV40 genomes were extracted 6 days after the infection from SV40 infected BSC-1 cells after the 5th serial passage with five μg of DNA analyzed on the neutral-neutral 2D gel, panel **A** shows the EtBr staining of this DNA and panel **B** shows the picture of a similar gel, containing 0.5 μg defective SV40 DNA following blotting and hybridization to SV40 probe and 4 hours exposure, panel **C**: 10 μg of extrachromosomal MNNG-treated CO60 DNA was analyzed under the same 2D gel conditions, blotted, hybridized to SV40 probe and exposed

for 24 hours, panel **D**: is a superimposition of panels B and C demonstrating that the arcs migrated identically, the four arcs are designated 1-4 throughout this application as marked in the figure;

FIGURE 3A-I is a structural analysis of the arc DNA, defective SV40 genomes were separated on 2D gel and DNA from arcs 1, 2 and 3 was extracted and examined by electron microscopy to reveal its structure (**C, F, I**), a sample from each DNA was mixed with the linear size marker λHindIII and re-run on 2D gels, the gels were blotted and were first hybridized to SV40 probe (**A, D, G**) and then to λ probe for reference of the linear DNA migration (**B, E, H**), panels **A**, **B**, and **C** correspond to arc 1 with **D**, **E** and **F** to arc 2 and with **G**, **H** and **I** to arc 3;

FIGURE 4A-B are photographs showing covalently closed circles form two arcs on 2D gels, (**A**) SV40 DNA extracted from viral particles and linear extrachromosomal DNA from treated CO60 cells was separated on 2D gel which was blotted and hybridized to SV40 probe, the three spots {1, 2, 3} represent the migration of the SV40 circular molecules and the smooth line corresponds to linear DNA, note that the faint spot {2} migrated on the first dimension the same distance as the lower spot {3}, on the second dimension, this spot migrated the same distance as the upper spot {1}, (**B**) is a 2D gel of extrachromosomal DNA from MNNG-treated CO60 cells in which arc 2 is invisible, demonstrating that under careful conditions during the preparation of the second dimension, the nicking of the closed circular DNA can be minimized;

FIGURE 5A-B are photographs of a neutral-neutral 2D gel of extrachromosomal DNA from MNNG-treated and from untreated control CO60 cells, extrachromosomal (Hirt supernatant) DNA (20 μg) prepared from untreated control CO60 cells 48 hours after seeding (**A**) and from MNNG-treated CO60 cells 96 hours after treatment (**B**) was analyzed by 2D gel electrophoresis, the two DNA samples

were run simultaneously in the same tank, blotted to the same membrane and hybridized together to the SV40 probe, the membrane was exposed for 17 hours;

FIGURE 6A-C are photographs of the separation of covalently closed circular molecules on CsCl-EtBr density gradient, one mg of extrachromosomal DNA from MNNG-treated CO60 cells was separated on CsCl-EtBr density gradient and two fractions were collected, a sample of each fraction was mixed with the linear size marker λHindIII and separated on 2D gels to determine the structure of the SV40 DNA, the gels containing the pool of the linear DNA and the nicked circles (**A**), and the pool of covalently closed circular DNA (**B**) were blotted and hybridized to SV40 probe, the arcs are marked in the corresponding numbers 1-4, the blot in panel **B** was also hybridized to λ DNA to visualize the migration of the linear DNA (**C**);

FIGURE 7 is a photograph showing identification of inverted repeats within the amplified circular SV40 DNA in MNNG-treated CO60 cells, covalently closed circular DNA (lanes 4 and 8) and total extrachromosomal DNA (lanes 2, 3, 6 and 7) were prepared from MNNG-treated CO60 cells and these DNAs were first linearized by *Bgl*I (which cleaves at the SV40 origin) and then were either directly cleaved by *Dpn*II (**-S1** lanes) or were subjected to the "snap back" assay prior to the *Dpn*II cleavage (**+S1** lanes), one ng of the *Alw*NI cut p2-C plasmid served as a control for the "snap back" assay (lanes 1 and 5), and as an internal control in the reactions which contained the cellular DNA (lanes 3, 4, 7 and 8), the DNA was separated on 1.3% agarose, blotted and hybridized to SV40 probe and p2-C: the plasmid fragments (1.8 and 1.55 Kb) consisting of SV40 sequences (lanes 1,3 and 4) and the 365 bp fragment containing inverted repeats (lane 5, 7 and 8);

FIGURE 8A-D are photographs of neutral-neutral 2D gel of extrachromosomal DNA from MNNG-treated and from

untreated control CHO cells, extrachromosomal (Hirt supernatant) DNA (30 μg) prepared from untreated control CHO cells (**A**, **A'**) and from MNNG-treated CHO cells (**B**, **B'**) was analyzed on 2D gel electrophoresis, following blotting, the DNA was hybridized to hamster Cot-1 probe, Short (16 hours, **A**, **B**) and long (48 hours, **A'**, **B'**) exposures of the membranes demonstrate the enhancement of circular molecules in the MNNG-treated CHO cells, the arrows point the arcs of relaxed circles (corresponding to arc 1 of SV40 DNA), amplified Extrachromosomal SV40 DNA from MNNG-treated CO60 cells was separated simultaneously in the same electrophoresis tank served as a reference for the migration position of the circle-arcs on the 2D gel following hybridization to SV40 probe (**C**), the typical arcs 1-4 are indicated, superimposition of panels **B'** and **C** indicates that the CHO arcs indeed migrated as supercoiled and relaxed circles (**D**);

FIGURE 9 is a photograph of neutral-neutral 2D gel analysis of HeLa DNA, the arrow points to the arc of relaxed circles;

FIGURE 10A-B are photographs of neutral-neutral 2D gel analysis of DNA from skin fibroblasts of (**A**) F-1651 cells (healthy donor) and (**B**) FA cells, where the arrow points to the arc of relaxed circles; and

FIGURE 11A-B are photographs of neutral-neutral 2D gel analysis of mitochondrial DNA (mtDNA) from CHO cells wherein (**A**) shows the results of a first hybridizing the DNA to Chinese hamster mtDNA and (**B**) is the same blot further hybridized to a λ probe and Cir indicates the circular and other non linear structures and complexes of mtDNA, open arrow represent the migration of the approximately 16 kb linearized mtDNA and full arrows represent circular molecules that were linearized upon the preparation of the second dimension.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

According to the present invention, a method of determining genomic instability is disclosed. The method includes the steps of isolating intact endogenous genomic DNA from a cell sample.

The isolated DNA is then analyzed by neutral-neutral (buffer pH) 2D gel electrophoresis and the presence of a population of heterogeneous incrementally sized circular DNA molecules is determined by the presence of the electrophoresis arc pattern as shown in the Figures. The presence of genomic instability is indicated by the presence of a specific, reproducible arc pattern exhibiting the incrementally sized circular DNA.

Separation of DNA on neutral-neutral 2D gel was performed according the procedure described by Brewer and Fangman [1987], which is incorporated herein by reference and as described herein below in the Examples. This procedure for the two-dimensional agarose gel electrophoresis is adapted from the procedure of Bell and Byers [1983]. The following modifications of the electrophoresis parameters were used: the first dimension was run in 0.4% agarose 1 V/cm (Fig. 4B, 8) or 0.5 V/cm (Fig. 2, 3, 4A, 5, 6) for 18 hours and the second, in 1% agarose 5 V/cm for 3.5 hours. The 2-D gel system is particularly suitable for the analysis of spcDNA, as it separates molecules according to both size and structure. In the first dimension, the gel is sparse, no EtBr is present and the DNA sample is run for a long time under low voltage. In the second dimension, EtBr is added, and the dense (~1%) gel is run under high voltage. Thus while the effect of molecular size is most pronounced in the first dimension, the effect of the molecular structure is maximized in the second dimension. The 2-D gel analysis provides both information on size and sequence content of the spcDNA, and the amount of spcDNA can be reliable quantitated.

The electrophoresis arc pattern showing the presence of a heterogeneous population of incrementally sized circular DNA molecules is best exemplified by Figures 5B and 8C. The pattern consists of generally three arcs and a fourth arc which is linear or linearized DNA. Arc 1 is opened circles (relaxed) DNA, arc 2 is covalently closed circles that were converted to a relaxed form. Arc 3 is covalently closed (supercoiled) DNA.

Probes for the repetitive sequences of the circular DNA can be used in the method of the present invention. As for example cot-1 for highly repetitive sequences or other probes for other cellular sequences can be used. These sequences can be for specific families of mid-repetitive sequences, for gene families or for telomeric sequences.

The DNA is isolated from a cell sample from the subject to be tested. Any standard method of isolating cells can be employed. The cell sample can be lymphocytes, skin fibroblasts or other cells that are readily isolated. Other readily isolated cells are cells from nasal or buccal smears. The subject can be any mammal including humans or veterinary significant mammals as well as other eukaryotic species that need to be examined for genomic instability.

Alternatively, the cells can be used directly without a specific DNA isolation step as set forth in Example 9 herein below. Briefly, the cells are formed into a block or plug of 1% low gelling temperature agarose, treated with Proteinase K and then utilized in the neutral-neutral 2-D gel electrophoresis.

The present invention further provides for a kit for determining genomic instability. The kit can include materials for isolating a cell sample and isolating DNA from the cell sample. The means for performing a neutral-neutral 2D gel electrophoresis and means for determining the presence of heterogeneous circular DNA molecules are also included in the kit. The means in the

kit can contain premade 2D gels (regular or pulse field), a flexible mold to hold gel for cutting and evaluating each arc. In addition reference samples of nuclear and mitochondrial DNA samples can be included as well as size markers for the circles.

Further, the kit can also include the means for preparing the cell plugs or blocks for use in the neutral-neutral 2D gel electrophoresis. This can include low gelling temperature (LGT) agarose, and an appropriate mold in which to form the plug or block.

The present invention also provides a method of screening for possible carcinogenic compounds by establishing a cell culture and exposing the cell culture to a compound which may be a potential carcinogen. DNA is then isolated from the cell culture, analyzed using neutral-neutral 2D gel electrophoresis. The presence of a population of heterogeneous incrementally sized circular DNA molecules is determined by the presence of the electrophoresis arc pattern. If circular DNA is present the compound is determined to be, at the least, a possible carcinogen and should undergo further testing.

In one preferred embodiment both nuclear and mitochondrial DNA is isolated and the presence of heterogeneous incrementally sized circular DNA molecules in each determined and compared. An increase in the number of circles in the nuclear DNA over the mitochondrial DNA compared to normalized reverence samples is used to determine an effect of the possible carcinogen. In general, the mitochondrial DNA is not affected by the potential carcinogen and therefore serves as the normalization parameter to the reference sample.

This method can be practiced using a cell culture system that is a chinese hamster SV40 transformed cell line or other cells such as a normal human fibroblast cell sample. Any cells or cell lines may be used in the practice of the present invention.

The present invention provides a method of determining a cancerous or pre-malignancy condition as well as if a patient is predisposed to premature aging. In the method, a cell sample is isolated from a patient and either analyzed directly or cultured. DNA is isolated from the cell sample and analyzed using the neutral-neutral 2D gel electrophoresis. If heterogeneous incrementally sized circular DNA molecules are determined to be present from the electrophoresis arc pattern then the cells are, at the least, indicated to be possibly either cancerous or pre-malignant or undergoing premature aging. Normalization can be practiced as described herein.

The present invention also provides a method of determining if a person is susceptible (i.e. has a genotype leading to susceptibility) to a given compound, such that they react to it as if it were a carcinogen. In the method a cell sample is isolated from a patient and cultured and then exposed to a compound to be tested, i.e. the carcinogen. The cell type chosen for testing will be determined based on the nature of the compound and the expected route of exposure as would be known to those skilled in the art. DNA is isolated from the cell sample and analyzed using the neutral-neutral 2D gel electrophoresis. If heterogeneous circular DNA molecules are determined to be present from the electrophoresis arc pattern after exposure to the compound, then the person being tested should be considered to have a genotype making them susceptible to the compound being tested, i.e. reacts to the compound as if it were a carcinogen. Normalization can be practiced as described herein.

Further, the present invention also provides a method for determining if a person has been possibly exposed to a carcinogen. In the method a cell sample is isolated from a patient and cultured. The cell type chosen for testing will be determined based on the nature of the compound and the route of probable exposure as

would be known to those skilled in the art. DNA is isolated from the cell sample, both nuclear and mitochondrial, and analyzed using the neutral-neutral 2D gel electrophoresis. If heterogeneous circular DNA molecules are determined to be present from the electrophoresis arc pattern and are greater in the nuclear sample than the mitochondrial sample, then the person has probably been exposed to the carcinogen at levels sufficient to induce spcDNA measurable by neutral-neutral 2D gel electrophoresis. Normalization would be practiced as described herein.

The method of the present invention demonstrates that supercoiled circles, relaxed circles and linear molecules migrate in distinct arcs on 2D gel electrophoresis. As shown in Examples 1 and 2, the present invention adapted the neutral-neutral 2D gel electrophoresis that is usually used for the detection of replication intermediates and for the identification of origins of replication [Brewer and Fangman, 1987] to identify circular DNA. As a model for circular DNA, a heterogeneous population of defective SV40 circular genomes was used. Their migration pattern on the 2D gels revealed four distinct arcs containing the SV40 sequences (Fig. 2B). The arc's patterns were different from those reported for replication intermediates of linear or circular molecules [Brewer and Fangman, 1987; Sundin and Varshavski, 1980] and represent different forms of a heterogeneous circular population.

The large yield of the defective viral genomes enabled the detection of these arcs by EtBr staining of the gel (Fig. 2A) and their elution for further identification by electron microscopy (EM). EM analysis clearly showed that the "arc DNA" represented different forms of circular molecules. The DNA eluted from arcs 1 and 2 contained mainly relaxed circles and small amounts of linear DNA that probably resulted from molecules which broke during the DNA preparation (Fig. 3C and 3F).

Reanalysis on 2D gels of the "arc DNA" eluted from both arcs 1 and 2 reconstructed arc 1 (Fig. 3A and 3D), demonstrating that both arcs 1 and 2 contained relaxed circles. Further analysis presented in Figure 4, clearly demonstrated that arc 2 represented molecules that migrated on the first dimension as supercoiled molecules and were nicked and converted into relaxed forms during the preparation of the second dimension. According to the EM analysis, the DNA eluted from arc 3 contained supercoiled circles as well as relaxed circles and linear molecules (Fig. 3I). This finding was confirmed by 2D gel analysis of the eluted DNA in which all the four arcs appeared (Fig. 3G). Since arcs 1 and 2 were shown to contain relaxed circles, then arc 3 consisted of supercoiled circles and that the other forms were generated due to nicking and breakage events. This interpretation was supported by the finding that DNA from arc 3 could be converted to form the three other arcs but the opposite (i.e. the conversion of DNA from arcs 1 or 2 to arc 3) never occurred. In addition, similar observations in which partially *in situ* digested supercoiled pBR322 molecules (separated on the first dimension) were converted into the relaxed form [Schvartzman et al., 1990] are in accordance with this interpretation.

As shown in Example 3 herein below, amplification of extrachromosomal circles is induced by treatment of CO60 cells with a carcinogen, MNNG. The separation of the MNNG treated-CO60 extrachromosomal DNA on the 2D gels revealed a similar arc pattern as the defective SV40 genomes (Fig. 2C and 2D). Densitometric analysis of several 2D gels showed that a significant fraction (ca. 30-50%) of the extrachromosomal SV40 sequences is found in the circles-arcs.

Comparison of the 2D patterns of identical amounts of extrachromosomal DNA from MNNG-treated and from untreated control CO60 cells revealed a dramatic increase

in the circular SV40 molecules following carcinogen treatment. This is a heterogeneous population of molecules with a wide size spectrum that was estimated to be <2 to >10 kb. The carcinogen treatment appears to activate mechanisms associated with the production of circular molecules.

The majority of the amplified SV40 sequences were previously shown to be associated with the extrachromosomal DNA fraction [Cohen et al., 1994]. Note that when the high-molecular-weight fraction (Hirt - pellet) from the treated CO60 cells was analyzed by the 2D gel, an arc pattern typical to circular molecules was observed, thus demonstrating that the high molecular weight DNA fraction was contaminated with low-molecular-weight circular SV40 DNA (data not shown). This finding further supports the idea that the SV40 amplification products are mainly extrachromosomal circles.

The circles can be produced by different mechanisms [Schimke et al., 1986, for reviews see references Schimke, 1988; Wahl, 1989 and Windle and Wahl, 1992]. Breakage events at arrested replication forks followed by ligations were suggested to generate circular molecules containing IRs as early amplification products [Windle et al., 1991]. Using the *in vitro* amplification system, applicants showed arrested replication forks during SV40 replication [Cohen et al., 1994]. The formation of hairpin and stem-loop structures in this system led applicants to propose the "U turn" replication model for their synthesis at arrested forks. Further secondary events might convert them into circular dimers containing IRs. Therefore using the present invention applicants searched for IRs within the circular SV40 DNA population in the treated CO60 cells as shown in Example 4 herein below.

Extrachromosomal circles containing IRs were found amongst the SV40 amplification products. CsCl-EtBr

density gradient of DNA from MNNG-treated CO60 cells was used to separate supercoiled circles from relaxed circles and linear extrachromosomal DNA. Using the 2D gel analysis the isolated DNA contained supercoiled molecules (Fig. 6B). A fraction of this DNA harbored IRs as demonstrated by the "snap back" assay and hybridization to SV40 probe (Fig. 7). The S1-resistant DNA represented only a small fraction of the circular molecules, suggesting that this fraction could be generated by the "U turn" replication. Possibly a larger fraction of the circles contain IRs, however, in these molecules the IRs might compose only a part of each circular molecule. Such configuration could be formed if the original "U turn" product was in the structure of stem-loop. In this case only the stem region appears in IRs while the loop regions is unique and would be digested following the "snap back" assay. This molecular organization can explain the reduction in the SV40 hybridization intensity to the S1 resistant DNA in comparison to the input DNA and in comparison to p2-C in which IRs are present in each molecule (Fig. 7, +S1 lanes in comparison to -S1 lanes). However, the primary amplification products *in vitro* could not be determined as to whether they were hairpins or stem-loop structure.

Using model hairpin and stem-loop molecules applicants found that on neutral-neutral 2D gels hairpins migrate in the position of linear double stranded DNA, while stem-loop molecules migrate faster, on the second dimension, forming an arc below the linear double stranded DNA. Such an arc was not detected under the assay conditions.

A better approach for the detection of hairpin and stem-loop structures is the neutral-alkali 2D gel which was previously described [Cohen et al., 1994]. In preliminary experiments applicants failed to detect such molecules in the DNA used in these experiments (preparations of 96 hours post treatment). However,

positive results were obtained in DNA prepared at short times after treatment. If synthesized as in the cell free system, hairpins and stem-loop structures do not seem to accumulate in the cells but rather further processes including circularization and degradation and therefore can not be easily detected. Note that p2-C was cloned from carcinogen treated CO60 cells and contains a stem-loop structure.

The amounts of IRs described in this application were smaller than those reported earlier [Cohen et al., 1994]. In the Examples, the DNA was first linearized with *Bgl*I a procedure that was found to reduce the amount of IRs both in the circular fraction and in the non fractionated extrachromosomal DNA. A possible explanation to this observation could be that the *Bgl*II linearized DNA which was examined in our previous study contained covalently closed circular molecules that reannealed upon the "snap back" assay, although they did not contain IRs. On the other hand, in this study, all the SV40-containing circles were linearized, only IRs could reanneal, and therefore, the signal decreased. This point was further strengthen when the amount of S1 resistant DNA obtained from *Bgl*I digested was compared to uncut DNA from MNNG-treated CO60 cells following the "snap back" assay. The amount of the S1 resistant DNA was dramatically reduced in the BglI-digested DNA (data not shown). The entire viral genome appears to be represented in IRs and display the same pattern and relative amount as do the total extrachromosomal fraction. Thus, the "U turn" model could be one of the mechanisms for circle formation.

Examples 5-7 show that there is enhancement of circular DNA (i.e., genomic instability; generation of spcDNA) in response to carcinogens and in malignancy. In example 5, DNA from CHO cells was analyzed using the 2D gel electrophoresis in order to identify spcDNA by this technique and to compare the cellular circles with the

SV40 amplified circular DNA. Based on pervious reports on spcDNA, Cot-1 DNA was used as a probe for the identification of circles containing highly repetitive sequences. Indeed, arcs that represent the circular DNA were detected following hybridization, besides a massive arc of linear DNA (Fig. 8A, 8A'). These results indicate the presence of a significant basal level of circular molecules in the CHO cells which can be detected by the 2D gels. The circular population in the CHO cells has an identical size range as the amplified circular SV40 DNA according to their similar arc length.

Analysis of spcDNA libraries from 3T6 cells [Sunnerhagen et al., 1986], mouse thymocytes [Fujimoto et al., 1985], and from human cells [Assum et al., 1993; Hollis and Hindley, 1986; Jones and Potter, 1995] revealed the enrichment of specific repetitive DNA families within the spcDNA relatively to their chromosomal abundance. Hence, these findings suggest the possible role of specific repetitive sequences in the formation and the selection of the circular molecules. The use of other probes such as specific families of mid-repetitive sequences, or gene families for hybridization to the DNA separated on the 2D gels, and/or telomeric sequences will display interesting hybridization patterns and illuminate the features of the spcDNA phenomenon in the aspects of both size and sequence content.

To test the effect of carcinogens, CHO cells, as well as normal human fibroblasts and cells from Fanconi's Anemia patients, were treated with MNNG in the same conditions as for the CO60 cells (for the detection of SV40 amplification). The human cells were treated with MNNG at a dose that caused 50% lethality (LD_{50}). The DNA from the treated CHO cells displayed elevated levels of circular molecules (Fig. 8B, 8B') in comparison to the untreated cells, as determined by the stronger hybridization to the circular-DNA arcs. The rate of

enhancement is lower than the amplification of the SV40 molecules in the CO60 cells, but, it is similar in principle. It should be noted that different treatment conditions on CHO cells might give different or more sharp results regarding the induction of circles. The SV40 system serves as an extreme model, due to the activation of the viral origin, and the initiation of DNA replication in a non-cell-cycle-depended-manner which could play a role in the amplification. However, the increase in the circular population is observed also in the cellular DNA of CHO cells, and the other cell types, upon carcinogen treatment and it does not depend on a viral origin. Thus the SV40 can serve as a model for this cellular phenomenon. Further, MNNG treatment enhanced the formation of the circular molecules, similarly to its effect on the SV40 sequences in the CO60 cells.

It has been reported that treatment with cycloheximide and drugs arresting DNA replication such as hydroxyurea (HU) and 7,1-dimethilbenzanthracene (DMBA) plus naladixic acid enhanced the formation of spcDNA [Sunnerhagen et al., 1989; Sunnerhagen et al., 1986]. Similar conditions were found to enhance the SV40 amplification in the CO60 cells [Lavi, 1982; Lavi and Etkin, 1981] and were reported to induce cellular gene amplification in different cell lines [Schimke, 1988]. It has been observed that higher degrees of genetic instability and increased levels of spcDNA generally paralleled each other [Motejlek et al., 1991; Motejlek et al., 1993; Riabowol et al., 1985; Wahl, 1989].

Using the 2D gel electrophoresis, extrachromosomal circles containing genomic sequences were detected in Chinese hamster lines (CO60 and OD4 cells) and in HeLa cells (Example 6). In addition, spcDNA was easily detected in cells from Fanconi's Anemia (FA) patients while they were not detected in fibroblasts from healthy donors. Upon carcinogen treatment, an induction of

spcDNA was observed in the normal fibroblasts and clear enhancement in circular population was detected in the FA cells. (Example 7)

The neutral-neutral 2D gel analysis of the present invention can serve as a very useful tool for the detection, isolation and analysis of spcDNA in various cell lines and in normal and tumor cells under different growth conditions as well as in cells under stress. This technique facilitates the direct characterization of the DNA included in the circular population and the detection of the sequences that are enriched and might be involved in their generation and selection.

As shown in Example 10, spcDNA has been shown to include telomeric sequences. This specific form of spcDNA has been termed tel-spcDNA. As with spc DNA, the tel-spcDNA increases on exposure to carcinogens and on aging. It therefore is also a measure of genomic instability.

Genomic instability is associated with and can be measured by extrachromosomal circular DNA and in particular spcDNA. Extrachromosomal circles were shown to be involved in early events of gene amplification both in mammalian cancerous cells [Carroll et al., 1988; Dolf et al., 1991; Nonet et al., 1993; Passananti et al., 1987; Ruiz et al., 1989; Ruiz and Wahl, 1988; von-Hoff et al., 1990; von-Hoff et al., 1988; Wahl, 1989; Windle et al., 1991; Windle and Wahl, 1992] and in lower eukaryotes [Beverley et al., 1984; Gardner et al., 1993; Grondin et al., 1993; Hanson et al., 1992; Hightower et al., 1988; Huber et al., 1989; Kapler et al., 1990; Papadopoulou et al., 1993; Petrillo-Peixoto and Beverley, 1988; Tripp et al., 1992; White et al., 1988]. These are usually large circles containing amplicons ranging in size from few tens to few hundreds kilobase pairs, which are often organized either in an inverted or direct order. A well-examined example is the amplification of the H circle in several species of *Leishmania*, which

accompanies the DHFR amplification [Beverley et al., 1984; Grondin et al., 1993; Kapler et al., 1990; Papadopoulou et al., 1993; Petrillo-Peixoto and Beverley, 1988; White et al., 1988]. These circles are usually about 30 Kb long, [Kapler et al., 1990] and can be found in wild type strains [Hightower et al., 1988] or can arise in response to Methotrexate selection [Papadopoulou et al., 1993]. The 4300 fold amplification of the ADA gene in circular molecules in the mouse fibroblast line B-1/50 was extensively characterized [Nonet et al., 1993]. This is a large circle of 500 Kb that is organized as an imperfect inverted repeat.

In the Examples set forth herein below, the IRs-containing circles are smaller than those described above, however one should consider that no selection was used and that the total crude amplification products were analyzed, which might include a minor fraction of larger molecules. These molecules might not be detected under the experimental conditions set forth below. Alternatively, the small sized circles might evolve to larger molecules by secondary recombination events [Carroll et al., 1988; Dolf et al., 1991; Hahn et al., 1992; Nonet et al., 1993; Ruiz et al., 1989; Ruiz and Wahl, 1988; von-Hoff et al., 1990; von-Hoff et al., 1988; Wahl, 1989].

Circular (spc) DNA (small sized extrachromosomal circles) is very common in a large variety of eukaryotic cells and were present in every examined cell line [for reviews see references Gaubatz, 1990; Rush and Misra, 1985; Yamagishi, 1986]. For example, spc DNA was found and cloned in human cells [Assum et al., 1989; Assum et al., 1993; Krolewski et al., 1984; Krysan et al., 1989; Krysan and Calos, 1991; Kunisada and Yamagishi, 1984; Kunisada and Yamagishi, 1987; Misra et al., 1989; Misra et al., 1987; Motejlek et al., 1993] monkey BSC-1 cells [Bertelsen et al., 1982; Schindler and Rush, 1985], Chinese hamster ovary (CHO) cells [Stanfield and

Helinsky, 1984] mouse cells [Flores et al., 1988; Fujimoto et al., 1985; Sunnerhagen et al., 1989; Sunnerhagen et al., 1986; Yamagishi et al., 1983] and *Drosophila melanogaster* cells [Stanfield and Helinski, 1976]. Therefore, spcDNA may represent a universal phenomenon in higher organisms. It is well accepted that they are derived from pre-existing chromosomal sequences and that they interact with each other [for a review see reference Gaubatz, 1990]. The diversity in their size, amount, sequence content and organization imply for the existence of several modes for their formation. They were suggested to be the by-products of specific chromosomal events reflecting genetic plasticity, such as recombinations [Jones and Potter, 1985; Riabowol et al., 1985; Sunnerhagen et al., 1986], transpositions [for a review see reference, Rio, 1990] or autonomously replicating DNA [Ruiz et al., 1989; Schimke, 1988; Wahl, 1989]. Their size ranges mostly between few hundred base pairs to few kilobases and cloning experiments showed a wide representation of repetitive sequences. In addition, unique chromosomal sequences were included in these circles as well [for reviews see references Gaubatz, 1990; Rush and Misra, 1985 and Yamagishi, 1986].

Analysis of spcDNA library derived from angiofibromas in patients with tuberous sclerosis revealed some unique cellular sequences that were proposed to be involved in duplications and rearrangements in the patients' genome [Assum et al., 1989]. Furthermore, a transposon-like sequence, which contains the R-repeats organized in an inverted orientation was found in a clone from spcDNA library of mouse thymocytes [Fujimoto et al., 1985]. A study on the DNA-repair-defect syndrome Fanconi's anemia showed the association of genetic instability and elevated levels of spcDNA molecules [Motejlek et al., 1993].

The CO60 (which are SV40-transformed CHE cells) model system used in the Examples herein below seems to

relate both to DNA amplification and to circular DNA formation. Gene amplification is well accepted as a marker for genomic instability characterizing transformed cells (as opposed to normal cells) and as part of tumorigenesis. spcDNA is a naturally occurring phenomenon which demonstrates the flexibility of the genome though its level is enhanced in situations of loss of genomic stability which is either endogenous, such as cancer and aging or exogenous such as exposure to carcinogens.

These two phenomena are not necessarily separated. One might lead to the other or there could be common mechanisms which are responsible for them both. Not only this model system seems to link between gene amplification and spcDNA. Common mechanistic principles proposed previously that spcDNA, which carry a selective advantage and contain an origin of replication, would remain in the cells, similarly to amplified sequences. As a working hypothesis, Rush and Misra [Rush and Misra, 1985] proposed that some members of the spcDNA represents amplified genes whose replication and expression are favored under appropriate conditions. An implication associated with this proposal is that the initial stages of the extrachromosomal amplification are not rare, but the detection of the amplified sequences requires selection to increase these molecules above the low number of cells containing these sequences. This is exactly what is shown for the SV40 amplification system: the circular molecules which are generated, will disappear if they do not confer a selective advantage. DNA amplification as revealed by drug selection of resistant cultured mammalian cells can involve extrachromosomal forms [Schimke, 1988] and may appear initially as unstable circular intermediates [Beverley et al., 1984]. Cellular oncogenes are also often amplified in extrachromosomal forms and are maintained in the tumor cells due to the growth advantage conferred to the cells

with such amplification. As mentioned above, these amplified sequences were probably slightly different from the original amplicon.

Gene amplification and spcDNA might arise from "U turn" replication or a replication-driven "onion-skin", which represents successive initiation events from a single origin. Alternatively, breakage and ligation events at arrested replication forks could also occur. The amplification of the circular molecules could result from unequal mitotic segregation coupled with selection or relaxed type of episomal replication, provided that an origin was captured in the circular molecules. Not only that the spcDNA formation reflects genomic instability, but further processing, such as autonomous replication and recombination events with each other or with the chromosomes, might contribute to additional genomic changes. Autonomous replication of spcDNA still has to be proven, and it is clear that these molecules have to contain origin features or other features that will enable replication such as telomeric sequences. However, there is some evidence that outside the chromosomal context, any human sequence included in a large enough viral circle (which is replication defective itself), could autonomously replicate [Krysan et al., 1989; Krysan and Calos, 1991]. Moreover, even without autonomous replication, the presence of such a heterogeneous reservoir of extrachromosomal circles is just prone to undergo secondary interactions such as homologous and nonhomologous recombination events to the chromosomes leading to clastogenic effects and thus it influences genomic stability. It should be noted that analysis of the integrated SV40 in subclones which were derived from MNNG-treated CO60 cells revealed new unique bands which differed from one clone to another. This finding suggests rearrangements of the viral sequences which could arise from reintegrations of extrachromosomal molecules [Lavi and Etkin, 1981a].

spcDNA may be a mechanism of gene amplification that has been maintained in evolution to respond to selective changes in the environment. The SV40 amplification system, which generates molecules in the same size and structure as spcDNA, strongly suggests that these processes are highly related.

The neutral-neutral 2D gel analysis presented here serves as an easy tool for the identification of chromosomal plasticity and for the characterization of sequences facilitating autonomous replication. In addition, other circular molecules such as mitochondrial DNA, plasmids and viruses can be studied using this approach.

The above discussion provides a factual basis for the use of neutral-neutral 2D gel electrophoresis to identify genomic instability and thereby have a method of early detection of malignant cells and a method of identifying potential carcinogens as well as providing a system for identifying aging associated disease. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

GENERAL METHODS:

General methods in molecular biology:

Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Springs Harbor Laboratory, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989). Electrophoresis buffers were according to Sambrook et al.

Cells and DNA preparation

CO60 cells [Lavi and Etkin, 1981], CHO cells and BSC-1 cells were propagated in monolayer cultures in

Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Biolabs, Jerusalem, Israel) as were all other primary cell isolates and cell lines utilized. Other primary cells utilized were F-1748 (normal human skin fibroblasts), K-277 and F-59 (human skin fibroblasts from FA patient). Other cell lines were HeLa (from ATCC), C631 (a CO60 subclone which maintains high levels of SV40 amplification under MNNG treatment; Lavi unpublished), CHE (chinese hamster embryo primary cultured cells).

Chromosomal (HMW, high-molecular- weight) and extrachromosomal (LMW, low-molecular-weight) cellular DNA was prepared according to the procedure described by Hirt [1967]. When tissue was used (instead of cells) frozen tissue samples were first ground under liquid nitrogen, by mortar and pestle. The fine powder was dissolved in Hirt lysis buffer [Hirt, 1967], and subsequent steps were performed as before. The amount of DNA was estimated by spectrophotometer (Gilford) at 260 nm.

Human and hamster Cot-1 DNA was purchased from Gibco BRL (Gaithersburg, MD, USA). 20-50 ng DNA were used per probe, depending on the labeling kit used.

Generation of defective SV40 genomes

BSC-1 cells were plated at a density of 5×10^6/14-cm-diameter plate and were infected with wild-type SV40 strain 777. Defective viral stock was prepared as described by Lavi and Winocour [Lavi and Winocour, 1972] in which SV40 was serially passaged in BSC-1 cells at high multiplicity of infection (m.o.i.). In the first few passages, a cytopathic effect (c.p.e.) appeared 4-5 days after infection. This time period increased with the next passages suggesting the generation of defective virus particles. In the 5th passage, c.p.e. was detected after 11 days, and therefore cells were infected with the 4th passage virus and harvested 6 days after infection. Viral DNA was prepared

from the infected cells by the Hirt procedure [Hirt, 1967].

MNNG Treatment

CO60 and CHO cells were treated with MNNG 24 hours after being seeded as was previously described [Berko-Flint, 1990]. Log phase cells were plated at a density of 5×10^6/14-cm-diameter plate. After 24 hours, the cells were treated with 10μg/ml MNNG (N-Methyl-N'-nitro-N-nitrosoguanidine; Aldrich), which had been freshly dissolved in dimethyl sulfoxide (Sigma), and added to the growth medium. One hour after the treatment, the medium was replaced with fresh medium and the cells were allowed to grow in carcinogen-free medium until they were harvested (72-96 hours post treatment).

Human fibroblasts (F-1748) were treated with 5 μg/ml MNNG. This concentration was determined as follows: F-1748 cells, seeded in a 24-well plate, were treated with a series of MNNG concentrations ranging from 1 μg/ml to 10 μg/ml (in duplicates) and the toxic effect of the treatment on the cells during 5 days was estimated. Treatment with 10 μg/ml MNNG was highly toxic 24 hours post treatment, however upon treatment with 5 μg/ml most of the cells were viable though changes were observed in their shape (i.e., some of the treated cells lost their regular elongated shape) demonstrating the effect of treatment. In lower concentrations no toxicity was observed and the structural changes were reduced as well (data not shown). Therefore, treatment with 5 μg/ml MNNG for further experiments was chosen.

Neutral-Neutral 2 dimensional gel

Separation of DNA on neutral-neutral 2D gel was performed according the procedure described by Brewer and Fangman [1987], which is incorporated herein by reference, with the following modifications of the electrophoresis parameters: the first dimension was run in 0.4% agarose 1 V/cm (Fig. 4B, 8) or 0.5 V/cm (Fig. 2,

3, 4A, 5, 6) for 18 hours and the second, in 1% agarose 5 V/cm for 3.5 hours.

First dimension: LMW DNA samples were mixed with 375 ng of λ/*Hind*III size marker and separated on EtBr-free 0.4% agarose gel in 1× TBE, at low voltage (approximately 1 Volt/cm, usually 20-30 Volts per 20 cm gel) overnight. The gel was then stained with EtBr, by gentle shaking for 20 minutes at room temperature in 0.3 μg/ml EtBr 1× TBE buffer.

Second dimension: The stained gel was illuminated by UV light, photographed, and the lane of choice was cut. This step is done as briefly as possible, to minimize relaxation of supercoiled circular molecules by EtBr under UV light. The cut lane was placed in a clean gel support at 90° rotation to the first electrophoresis direction. The lane was cast with 0.8-1% agarose (cooled to ~60°C in 1× TBE that contains 0.3 μg/ml EtBr), and the gel was allowed to quickly set at 4°C. 1× loading buffer was added at the start of the second dimension electrophoresis. The second dimension was then run in 1× TBE buffer that contains 0.3 μg/ml EtBr, in a cold room, at 5 Volts/cm for 3.5-4 hours.

Gel blotting: After electrophoresis, the gel was again photographed. Then, acid de-purinization was performed by shaking the gel in 0.2 M HCl solution for 20 minutes. The gel was then washed twice with double-distilled water, and was denatured for 40 minutes in 0.4 M NaOH solution. The denatured gel was dry-blotted, according to the procedure described by Church and Gilbert [1984]: 0.4 N NaOH solution served as transfer buffer, and the gel was blotted upside-down onto a Hybond-N' nylon membrane (Amersham, Amersham, UK) overnight. Next, the membrane was washed with 2× SSC and dried on Whatman 3 MM chromatography paper (Whatman International Ltd., Maidstone, UK). The DNA was fixed to the membrane by cross-linking under 70 mJ/cm^2 UV irradiation with Amersham's 2500 UV cross-linker

(Amersham, Amersham, UK), followed by baking for 2 hours at 80°C.

Normalization of DNA amount

To normalize the signals that were obtained in the gels, blots are reprobed with a human mitochondrial DNA (mtDNA) probe. Mitochondrial DNA quantities are reported to remain unchanged in carcinogen treated cells [Sunnerhagen *et al.*, 1989]. As it is purified with the LMW DNA, it serves as a constant parameter for the total amount of DNA that is loaded onto the gel and as a marker for the integrity of the circular DNA in the DNA sample. mtDNA migrates on the 2D gels in a typical pattern that can be easily distinguished from the linear DNA and from the arcs of the circular smaller molecules. Quantitative analysis by PhosphorImager of the mtDNA signals showed that mtDNA quantity in the control DNA sample was twice that in MNNG treated-cell samples. Therefore, the induction of the cellular circular DNA by MNNG treatment, is even stronger than demonstrated.

Preparation of DNA for electron microscopy

50μg of extrachromosomal DNA containing the defective SV40 genomes was separated on the 2D gel. The four arcs were visualized by EtBr staining and DNA from arcs 1-3 (not including arc 4 which contained the linear DNA), was extracted from the gel using Elutip-D columns (Schleicher and Schuell, Dassel, Germany) according to the manufacturers instructions. The DNA samples were resuspended in 50μl TE and examined by EM as previously described [Kleinschmidt and Zahn, 1959]. A sample from each eluted DNA was mixed with λ *Hind*III linear size marker and rerun on a 2D gel to define its arc pattern following the extraction process. The gels were blotted to Hybond-N nylon membrane and hybridized to SV40 probe and to λ probe.

CsCl Ethidium-Bromide density gradient

CsCl Ethidium-bromide density gradient was used to separate the supercoiled circles from the linear DNA and

from the relaxed circles according to the procedure described by Sambrook et al. [1989]. One mg of extrachromosomal DNA from MNNG-treated CO60 cells was loaded onto the gradient which was run in Beckman ultracentrifuge (Ty. 65 rotor; Beckman) in 40000 rpm at 20°C for 42 hours. The fast migrating portion of the gradient, consisting the supercoiled molecules, was collected in aliquots and the DNA containing fractions were pooled following determination by UV illumination. The lower density band containing the linear molecules and the relaxed circles was collected separately. The structural identity of the SV40 DNA in the two pools was determined by the neutral-neutral 2D gel analysis as described in the Examples (Fig. 6).

The "snap back" assay: identification of IRs

Five μg of the supercoiled DNA fraction from the CsCl density gradient was linearized by *Bgl*I, mixed with 1 ng of *Alw*NI digested p2-C (an inverted repeat-containing plasmid) and subjected to the "snap back" assay as described by Ford et al., [Ford et al., 1985; Ford and Fried, 1986]. In brief, the linearized DNA was denatured at room temperature with 50 mM NaOH for 60 minutes, the volume was increased to three-fold the initial volume with ice-cold water and then the DNA was neutralized with 1 M HCl and 1 M Tris HCl (pH 7.8) on ice. The DNA was digested with nuclease S1 (10 units/μg of DNA, Boehringer-Mannheim) in 50 mM NaCl-33 mM sodium acetate-0.03 mM $ZnSO_4$ (pH 4.5) for 30 minutes at 14°C, and the reaction was terminated with 10 mM EDTA and by phenol extraction. The S1 nuclease-resistant DNA was precipitated and analyzed with the restriction enzyme *Dpn*II. The DNA was separated on 1.3% agarose gels, blotted to a nylon membrane (Hybond-N, Amersham, Amersham, England) and hybridized to SV40 probe under the standard conditions.

EXAMPLE 1

EXTRACHROMOSOMAL CIRCLES OF HETEROGENEOUS SIZE CAN BE DETECTED BY NEUTRAL-NEUTRAL 2 DIMENSIONAL GEL ELECTROPHORESIS.

To determine whether circular molecules are included within the amplified SV40 DNA applicants searched for an easy and convenient approach to detect and to analyze such molecules. The separation of circular DNA on the CsCl-EtBr density gradient is commonly used. However, it requires the preparation of huge amounts of extrachromosomal DNA and is inefficient due to the loss of a significant fraction of the circular molecules - the relaxed circles - which migrate with the linear DNA. Moreover, this approach does not facilitate further characterization of the circular molecules other than cloning or electron microscopy.

Applicants adapted the neutral-neutral 2-dimensional (2D) gel electrophoresis assay previously described by Brewer and Fangman [1987] for the structural analysis of the amplified DNA accumulated in the carcinogen-treated CO60 cells. This assay is widely used for the separation of branched DNA molecules from linear ones according to their differential migration on these gels. In addition, it was previously shown that circular structures can be separated from the linear DNA on 2D gels since covalently closed and nicked circles had a retarded migration in comparison to linear molecules with the same mass [Oppenheim, 1981]. However, in these experiments the examined DNA contained only one or few species of extrachromosomal circles that migrated as discrete spots on the 2D gels [Martin-Parras et al., 1992; Oppenheim, 1981].

To study the migration pattern of a heterogeneous population of circular DNA molecules applicants chose to use defective SV40 genomes which were shown to be supercoiled and relaxed circular DNA of heterogeneous size [Lavi and Winocour, 1972]. Defective SV40 genomes

were produced (as described herein above) by serial passages of the virus in BSC-1 cells at high multiplicity of infection (m.o.i.). The viral DNA was extracted from the infected cells using the Hirt procedure [Hirt, 1967], and analyzed on 2D gel. Blotting followed by hybridization to SV40 probe revealed four arcs, marked 1-4 throughout this application as shown in Fig. 2B. Three of those arcs were detected by EtBr staining and are shown in Figure 2A, the fourth arc (arc 2) is very faint and was invisible in this picture. Arc 4 co-migrated with the λ *Hind*III linear size marker (Fig. 2A, see also Fig. 3B, 3E and 3H) thus representing linear DNA. The patterns of the other three arcs (arcs 1-3) were completely different from those formed by replicating intermediates of a single molecule or of a few molecules [Brewer and Fangman, 1987] as well as from the pattern of catenated molecules [Brewer and Fangman, 1987; Sundin and Varshavski, 1980], suggesting that these arcs represent populations of circular DNA. This arc pattern was not shown in the prior art references, as for example Brewer and Fangman [1987] and Oppenheim [1981].

To test whether such structures appear among the amplified SV40 DNA, similar 2D gel analysis was performed on 10μg of DNA from MNNG-treated CO60 cells prepared 96 hours post treatment. Only arc 4 (representing the linear DNA) could be detected by EtBr staining since it consisted mainly of broken cellular sequences as was determined by its strong hybridization to hamster repetitive DNA (Cot-1) probe (data not shown). However, upon hybridization to SV40 probe, all the four arcs appeared (Fig. 2C). The pattern of these arcs was similar to that of the defective SV40 circular genomes but the arcs were much longer thus representing a wider size distribution of circular DNA. Superimposition of Figures 2B and 2C (in which the gels were run simultaneously in the same tank) verified the

co-migration of the defective SV40 genomes with the CO60 arcs (Fig. 2D).

In further experiments several plasmids in the sizes of 2-8.2 kb were mixed with the CO60 extrachromosomal DNA. Upon 2D gel analysis the plasmids co-migrated with arcs 1-3 and hence, the size spectrum of the amplified SV40 circular DNA is <2 to >10 Kb (data not shown). Note that each of arcs 1-3 from the CO60 extrachromosomal SV40 DNA contains a single more intense spot which possibly represents a predominant extrachromosomal circular molecule existing in the CO60 cell line (for example see Figs. 2C, 4B and 5B).

EXAMPLE 2

ELECTRON MICROSCOPE ANALYSIS OF "ARC DNA".

For further analysis, the DNA from each of arcs 1-3 was gently purified from a preparative 2D gel of the defective SV40 circular genomes. A sample of each DNA preparation was mixed with λ size marker and was re-run on the 2D gels to asses the yield and the integrity of the purified molecules. The blots were hybridized to SV40 probe (Fig. 3A, 3D, 3G) and then to λ probe to reveal the position of the linear DNA (Fig. 3B, 3E, 3H). The remaining purified "arc DNA" was analyzed by electron microscopy (EM) to determine the structures of the molecules (Fig. 3C, 3F, 3I). EM analysis of the DNA eluted from arc 1 displayed nicked circles (Fig. 3C) and small amounts of linear DNA. This arc was reconstructed upon a second 2D gel analysis (Fig. 3A). In addition, a faint arc of linear DNA appeared (arc 4), probably resulting from breaks in the circular DNA. This demonstrates that arc 1 represents the nicked circles.

By reanalysis on 2D gel, the DNA purified from arc 2 could not reconstruct this arc (Fig. 3D). However, the arc and the EM picture were identical to those of arc 1 (Fig. 3D, 3E, 3F), demonstrating that arc 2 consisted of nicked circular molecules like those in arc 1. As

illustrated in Figure 4 and discussed below, the open circles resulted from nicking of the closed circular molecules during the preparation of the second dimension of the gel.

The DNA eluted from arc 3 yielded on 2D gel the original arc 3 as well as the three other arcs (Fig. 3G). EM analysis of this DNA revealed supercoiled molecules, open circles and linear molecules (Fig. 3I). In examination of 31 fields, 355 molecules were counted from which 41% were supercoiled, 32% relaxed and 27% linear. These numbers are in agreement with the data presented in Figure 3G. The data therefore shows that arc 3 originally contained covalently closed circles (supercoiled) which underwent nicking or breakage events during the purification procedure, probably due to the large amount of EtBr that intercalated into the DNA.

Analysis of wild-type SV40 genome and of several plasmids on 2D gels demonstrated that every examined circular molecule migrated on the first dimension as 2 bands representing the nicked (relaxed) and the supercoiled (covalently closed) circles. However, upon electrophoresis on the second dimension (Fig. 4A), the supercoiled DNA band separated into 2 spots: one migrated fast as expected for supercoiled molecules (Fig. 4A - 3) and the other (Fig. 4A - 2) migrated slowly to the same distance as did the relaxed circles (Fig. 4A - 1). This migration pattern suggests that the retarded spot (Fig. 4A - 2) represents a fraction of the original supercoiled circles that were nicked after the first dimension. EtBr staining and UV illumination could have caused nicks in the supercoiled molecules during the preparation of the second dimension, resulting in the formation of open circular molecules. Heterogeneous populations of such DNA molecules will generate arc 2. Note that arc 2 is faint and in some cases is invisible (Fig. 3G, and compare Fig. 4B and Fig. 5B), probably due to different nicking rates in the different experiments.

EXAMPLE 3

AMPLIFIED SV40 DNA IS CIRCULAR.

Having established the technology, applicants used the 2D gel analysis to determine whether the circular SV40-containing molecules are amplified following MNNG-treatment of CO60 cells. The 2D gel pattern was compared to the intensity of SV40 hybridization of 20 μg of extrachromosomal DNA from MNNG treated (Fig. 5B) and from untreated control (Fig. 5A) CO60 cells. The results show a dramatic increase in the extrachromosomal circular SV40 DNA in the treated CO60 cells.

Densitometric analysis of several blots containing low-molecular-weight DNA from MNNG-treated CO60 cells (including those in Figures 2C and 5B), indicate that the circular DNA comprises 30-50% of the total SV40 DNA. Thus, a significant fraction of the amplified SV40 DNA is organized in circles. These findings clearly demonstrate that circular molecules are the primary SV40 amplification products in the MNNG-treated CO60 cells.

It should be noted that the circular fraction was relatively large when the DNA was tested shortly after its preparation or after stored frozen, and decreased with time (especially the supercoiled DNA) following storage at 4°C, suggesting that a portion of the linear molecules were originally circular.

The specific induction of circular DNA in response to MNNG treatment in this system raises two questions. One is whether these circles contain inverted repeats as predicted by the "U turn replication mode". The second, which is probably more important, is whether circular molecules that contain cellular sequences are found in transformed cells and if they are induced upon carcinogen treatment.

EXAMPLE 4

IRs ARE DETECTED IN THE AMPLIFIED EXTRACHROMOSOMAL CIRCULAR DNA.

To test whether IRs are found within the amplified circular DNA population, extrachromosomal DNA was separated from MNNG-treated CO60 cells on CsCl-EtBr density gradient. Two pools of fractions were collected: one from the low-density and the second from the high-density portions of the gradient as described herein above. The 2D gel electrophoresis served as a tool for structural identification of the DNA. Samples from each pool were mixed with λ size marker, separated on 2D gels and hybridized to SV40 probe (Fig. 6). The first pool contained, as expected, arcs 1 and 4 which are the nicked (relaxed) circles and the linear DNA respectively (Fig. 6A). The second pool, containing the supercoiled circular molecules, yielded the two other arcs, arc 3 representing the supercoiled molecules and arc 2 displaying supercoiled molecules that were converted to relaxed forms during the preparation of the second dimension (Fig. 6B). The identity of these arcs in relation to linear DNA was verified by hybridization of the blot to λ probe (Fig. 6C). These findings indicate that the SV40 DNA in the high density pool consisted exclusively of supercoiled molecules.

The supercoiled fraction was examined for the presence of IR-containing SV40 sequences using the "snap back" assay [Ford et al., 1985; Ford and Fried, 1986]. This assay is based on the ability of inverted duplications to snap back following denaturation and rapid renaturation, into double stranded forms and thus resist the S1 nuclease hydrolysis. Figure 7 shows the results of the "snap back" assay on the closed circular fraction of extrachromosomal DNA from treated CO60 cells as well as on total extrachromosomal DNA from these cells.

As an internal control a plasmid (p2-C, [Cohen et al., 1994]) was used containing a duplicated segment of SV40 sequences which is organized in an inverted order as was determined by EM analysis and restriction mapping. Cleavage of p2-C with the restriction enzyme *Alw*NI generates three fragments (3.1, 1.8 and 1.55 kb) two of which (1.8 and 1.55 kb) contain SV40 sequences and therefore are visible following hybridization to SV40 probe (Fig. 7 lane 1). Upon the "snap back" assay the three original fragments disappeared and a new fragment of 365 bp consisting of the folded IRs appeared (Fig 7 lane 5).

Being aware of the presence of a large fraction of covalently closed circular molecules amongst the amplified DNA, the supercoiled fraction was linearized by *Bgl*I which cleaves at the SV40 origin of replication. Such digestion of the extrachromosomal DNA with *Bgl*I, abolished SV40 hybridization to arcs 1-3, demonstrating that these arcs contained molecules with the viral origin which were linearized by *Bgl*I (data not shown). The linearized molecules were mixed with *Alw*NI digested p2-C (the internal control) and then divided into two fractions. The first, containing 10% of the DNA, was directly digested with *Dpn*II to reveal the pattern of the SV40 DNA (Fig 7. lane 4). Note that the plasmid DNA (p2-C) was methylated and therefore was *Dpn*II resistant. The remaining 90% of the DNA was first subjected to the "snap back" assay and then to *Dpn*II digestion (Fig. 7, lane 8).

As a result, the S1-resistant DNA had the same SV40 pattern, though much weaker, as in the non S1-digested control. In addition a new p2-C fragment, containing the IRs, appeared. This result indicates that a fraction of the circular amplified SV40 DNA is arranged as IRs as predicted by the "U turn" model. When the same test was performed on total low-molecular-weight DNA from MNNG-treated CO60 cells, in the presence (Fig. 7 lanes 3

and 7) or the absence of the internal p2-C control (Fig. 7 lanes 2 and 6), the same *DpnII* pattern appeared following hybridization to SV40 probe. The proportions of the S1-resistant SV40 DNA in the supercoiled and in the nonfractionated extrachromosomal DNA were similar suggesting that the proportion of IRs in supercoiled DNA is similar to its proportion in the total low-molecular-weight DNA.

Applicants had previously shown that sequences representing the complete integrated SV40 genome were organized as IRs in the chromosomal and extrachromosomal DNA from MNNG-treated CO60 cells [Cohen et al., 1994]. The identical *DpnII* patterns of the total extrachromosomal DNA (Fig 7. lanes 2 and 6) and of the supercoiled fraction (Fig 7. lanes 4 and 8), before and after the "snap back" assay, demonstrate that the entire integrated SV40 genome is included in the amplified circular population and that at least a fraction of the circular viral molecules is organized as IRs.

EXAMPLE 5
CIRCULAR DNA MOLECULES ARE ENHANCED UPON MNNG TREATMENT OF CHO CELLS.

To investigate circular molecules of cellular sequences CHO cells were examined which were reported to contain small polydisperse (spc) DNA that was detected by other methods [Stanfield and Helinski, 1976]. Applicants asked whether spcDNA can be detected using the 2D gels, whether it would be similar in size to the amplified SV40 circles and whether MNNG-treatment has any effect on this DNA.

Extrachromosomal DNA was prepared from MNNG-treated CHO cells 96 hours post treatment, and from untreated control cells. Equal amounts of DNA from control and treated cells were analyzed on the 2D gel in the same tank as extrachromosomal DNA from MNNG-treated CO60 cells. The gels were blotted and since the whole genomic

sequences were reported to appear in spcDNA, applicants chose to use Cot-1 DNA, which represents the genomic repetitive DNA, as the cellular probe to detect such circles in the CHO DNA. Figure 8 shows short and long exposures of the blots containing the DNA from the untreated (panels A, A') and from MNNG-treated (panels B, B') CHO cells. The blot containing DNA from MNNG-treated CO60 cells was hybridized to SV40 probe to serve as a reference for the migration position of the arcs containing circular DNA (Fig. 8C). Comparison of the pattern of the amplified SV40 circular DNA (Fig 8C), to the hybridization patterns in panels A and A' shows that beside the massive arc of linear DNA (which corresponds to arc 4 in panel C), arcs of circular DNA are clearly observed (corresponding to arcs 1-2 in panel C). The arc that corresponds to arc 3, is masked by the heavy hybridization to the linear DNA. The arrows point to arc 1, as a representative for comparison between the panels. These results demonstrate the presence of a significant amount of circular DNA molecules in the low-molecular-weight DNA fraction of CHO cells and that they are characterized by a similar size range to the amplified SV40 circles. Note that the level of the circular molecules in the CHO cells, is lower compared to that of SV40 circles observed in treated CO60 cells and that most of the hybridization to the Cot-1 probe was to the linear DNA arc. This was, in fact, the expected result since most of the extrachromosomal DNA is linear and since the Cot-1 hybridization represents all the genomic DNA. On the other hand, SV40 hybridization in CO60 DNA, represents only the viral DNA which comprise a small fraction of the total extrachromosomal DNA. However, it is very common in the circular population due to its extremely massive amplification

Enhancement of the circular DNA population is observed following the carcinogen treatment of CHO cells. This is indicated by the increased hybridization to the

arcs of circular DNA in Fig. 8B and 8B' in comparison to the arcs in Fig. 8A and 8A' which contain DNA from the untreated cells. To quantitate the enhancement of the circular DNA in the carcinogen treated CHO cells in comparison to the control, the blots were reprobed with hamster mitochondrial DNA. Mitochondrial DNA is extracted with the low-molecular weight DNA which was previously shown to remain unchanged following carcinogen treatment [Sunnerhagen et al., 1989]. Thus, the mitochondrial signals were used to normalize the circular DNA signals in Fig. 8. Densitometric analysis revealed the circular DNA in carcinogen treated CHO cells was enhanced 3.85 to 4.11 fold (for the lower part of arc 2 and for the entire arc 1, respectively) in comparison to the circular DNA from the control cells. It should be noted that induction of circles containing Cot-1 DNA was observed in MNNg-treated CO60 cells.

Superimposition of panel C (Fig. 8) on panel B' (Fig. 8) confirmed that the arcs in the CHO blots migrated exactly to the same position as did the SV40 circles (Fig. 8D). Thus the data show that a significant amount of circular DNA is found in the hamster transformed CHO cell line and represents a characteristic feature of transformed cells which are known to be unstable. Furthermore, the circular DNA is enhanced in the carcinogen-treated CHO cells in a similar mode to the SV40 amplification in the treated CO60 cells.

EXAMPLE 6

EXTRACHROMOSOMAL CIRCULAR DNA IN OTHER CELL LINES

Using the 2D gel electrophoresis, extrachromosomal circles containing genomic sequences were detected in HeLa cells (Fig. 9). Extrachromosomal DNA was isolated from HeLa cells and analyzed. Following blotting, the gel was hybridized to a human Cot-1 probe and exposed for 24 hours. As noted by the arrow, an arc of relaxed circles is observed. The identity of the arc is verified

by comparison of its position to the migration position of the arcs of the amplified SV40 DNA from MNNG-treated CO60 cells as shown in Figure 5b. Similar results were obtained in an additional SV40 transformed human cell line (847) and in rat cells.

EXAMPLE 7

DETECTION OF CIRCULAR DNA IN PATIENTS AND FOLLOWING CARCINOGENIC TREATMENT

As shown herein above, circular DNA molecules create a typical reproducible arc pattern on neutral-neutral 2D gels. In order to obtain such a reference pattern in the following experiments, DNA from MNNG-treated CO60 cells was either separated simultaneously in the same electrophoresis tank as the examined cellular DNA or was mixed with the tested human DNA sample. This DNA contains a heterogeneous population of amplified circular SV40 molecules as shown above. Following blotting and hybridization to an SV40 probe, this DNA served as a reference for the migration position of the circular DNA arcs. When human DNA was mixed with the CO60 DNA, the blot was hybridized twice, once to the cellular repetitive DNA probe (Cot-1), and afterwards to the SV40 probe. The Cot-1 probe represents the highly repetitive genomic DNA. In these experiments the molecules that contain the repetitive sequences are visualized and thus represent the total genomic DNA. Note that the MNNG-treated CO60 DNA and the human Cot-1 probe do not cross-hybridize. Thus, mixing the two DNAs did not generate an artifact regarding the hybridization of the human Cot-1 probe to circular hamster DNA.

spcDNA was easily detected in cells from Fanconi's Anemia (FA) patients (Fig. 10B) while it was not detected in fibroblasts from healthy donors (Fig. 10A). An arc of relaxed circles is shown at the arrow in Fig. 10B.

For this Example, fibroblasts from a healthy donor and from an FA patient were propagated in culture for ten

and eight passages respectively. Extrachromosomal (Hirt supernatant) DNA was analyzed by 2D gel electrophoresis, blotted and hybridized to a human Cot-1 probe. The membranes were exposed for a week. The arc of relaxed circles was verified by comparison to the hybridization pattern of MNNG-treated-CO60 DNA (which was mixed with the human DNA prior to the 2D assay) to an SV40 probe (as shown in Fig. 5b). Similar results were observed upon examination of DNA taken from passage 11 and passage 7 of two additional patients.

To investigate the status of circular DNA in normal cells, normal human skin fibroblasts were taken from a healthy donor and were propagated in culture for a few passages. It was previously reported that the lowest amounts of spcDNA were obtained in normal human skin fibroblasts [Motejlek *et al.*, 1993; Kunisada *et al.*, 1985]. LMW DNA from skin fibroblasts of a healthy donor propagated in culture for 10 passages was analyzed by neutral-neutral 2-D gel. Upon blotting and hybridization to a human Cot-1 DNA probe, a massive arc of linear DNA was observed as well as a faint lower arc representing single stranded DNA. However, an arc of circular DNA could not be detected even after longer exposures (48 hours in PhosphorImager), and DNA from another healthy donor behaved similarly (data not shown). This result demonstrates that under the assay conditions of the present invention, circular DNA could not be detected in the normal human skin fibroblasts.

Normal skin fibroblasts (F-1748) taken from a healthy donor and propagated in culture for 10 passages, were treated with MNNG. Hirt supernatant DNA was prepared from control cells and from MNNG-treated (5 μg/ml) cells, 48 hours post treatment, and was analyzed on the 2D gels. 8.4 μg of DNA (from untreated and treated cells, respectively) were separated on the neutral-neutral 2D gel. Following blotting, the DNA was hybridized to a human Cot-1 probe and exposed to Kodak

sensitive AR film for 48 hours. After removal of the probe the blot was rehybridized to a human mtDNA probe and exposed to Kodak sensitive AR film for 3 days. DNA from MNNG-treated CO60 cells was mixed with the human DNA sample and hybridization of the blot to an SV40 probe and served as reference for the migration position of the arcs representing circular DNA.

In response to MNNG treatment of the normal skin fibroblasts, an arc of relaxed circles was observed. Hence, the exposure to MNNG induced the formation of circular DNA molecules in normal human skin fibroblasts which is not observed in control cells. The identity of the arcs generated upon hybridization with the human Cot-1 probe was determined by comparison to the pattern of the SV40 amplified DNA from MNNG-treated CO60 cells that was mixed with the human DNA samples (data not shown). This experiment demonstrates that circular molecules can be induced in normal human cells in response to MNNG-treatment.

Low molecular weight DNA was prepared from a human colon carcinoma and normal colon tissue and 80 μg each were analyzed by neutral-neutral gel electrophoresis. Following hybridization to the human Cot-1 probe, an arc of open circles appeared clearly as well as the heavy arc of the linear molecules. LMW DNA from MNNG-treated CO60 cells was mixed with the tumor DNA and served as an internal reference for the migration position of the circular DNA arcs. Reprobing of the blot with an SV40 probe revealed the typical arc reference pattern. Similar results were obtained upon analysis of DNA from several other human colon carcinomas.

In summary, upon carcinogen treatment, an induction of spcDNA was observed in the normal fibroblasts and clear enhancement in circular population was detected in the FA cells. Further, in a patient with colon carcinoma higher levels of spcDNA were observed in comparison to the DNA from the healthy colon tissue of the same

patient. In cell culture of both normal and transformed cell lines, spcDNA was induced in response to carcinogen (MNNG) treatment. [Cohen, et al., 1997]

EXAMPLE 8

GENOMIC INSTABILITY, spcDNA AND AGING

As discussed herein above, genetic rearrangements can be a part of the cellular differentiation pathways and may play a role in developmental and aging processes. To study this applicants studied changes in mitochondrial DNA during aging.

The 2D gel technique facilitates the analysis of mitochondrial (mt)DNA molecules whose size in mammalian cells in approximately 16 kb. Changes in the integrity of mtDNA as a result of aging or oxidative stress were extensively investigated. Characteristic deletions in the mtDNA were frequently observed in aged people and their percentage increased with age up to 100% in people over 80 years [Corall-Debrinski et al., 1992; Baumer et al., 1994; Yang et al., 1994; Torii et al., 1992; Katayama et al., 1991; Hayakawa et al., 1991]. Several lines of evidence indicate that mitochondrial genomic instability may be an important contributor to age-related degenerative diseases because of its crucial role in oxygen supply [Katsumata et al., 1994; Kowald and Kirkwood, 1993; Lessa et al., 1994; Goldstein and Shmookler Reis, 1984].

Applicants show herein that the 2D gel can be used for the detailed investigation of changes in the size of mtDNA (Fig. 11). Extrachromosomal DNA from CHO cells (30μg) was mixed with λ *Hind*III size marker, separated on 2D gels according to the protocols as detailed herein above and blotted. The DNA was first hybridized to Chinese hamster mtDNA probe (cloned ATPase and COX 3 genes) and exposed for 16 hours. The results are shown in Fig. 11A. The same blot was then further hybridized (on

top of the first probe) to a λ DNA probe to reveal the position of the linear size markers as shown in Fig. 11B.

A typical migration pattern was observed in mtDNA from CHO and chinese hamster embryonic (CHE) cells, consisting of a linear band in the size of 16 kb and a non-linear smear representing the circular structures. This smear was not completely analyzed but the horizontal lines between the spots seem to represent molecules with the same structure and the same mass (in the second dimension) that migrate to different positions in the first dimension. It appears that these structures represent complexed circular structures. It was reported that large multimer complexes of mtDNA were purified from HeLa cells as well as the two circular forms and the linear mtDNA [Higuchi and Linn, 1995].

The 2D gel assay can be used in the research of mtDNA in aged cells from human and laboratory animals and cells under oxidative stress. This new approach will contribute to the understanding of the alteration in mtDNA in normal aged cells, in stressed cells and in cells from patients suffering from mitochondrial disorders such as male infertility, artherosclerotic heart disease and others [Cummis et al., 1994; Corral-Debrinski et al., 1992; Tritschler and Medori, 1993].

EXAMPLE 9

METHOD TO ANALYZE CELLULAR DNA DIRECTLY BY NEUTRAL-NEUTRAL 2D GEL ELECTROPHORESIS

As described hereinabove the neutral-neutral 2D gel requires the isolation of DNA and loading of it into the gel. This step is time consuming and when limited numbers of cells are available can be a limiting step. Applications have therefore improved the method of the neutral-neutral 2D gel electrophoresis to incorporate the isolation of the DNA from the cells in the gel itself.

1. Cells in culture are washed once with 1× PBS, and then incubated with 4 ml trypsin (per 14 cm diameter

plate) at 37°C for 5-15 minutes. The trypsin solution is removed, the cells counted and collected by centrifugation at 2000 rpm for 5 minutes at 4°C in a Sorvall RT6000 desktop centrifuge with a H1000B rotor. Cell suspensions are pelleted as above without the trypsination step. The cell pellet is washed once with SE (75 mM NaCl, 25 mM Na-EDTA, pH 7.4) buffer and re-suspended in SE at 4 x 10^7 cells/ml, at room temperature.

2. Melted 1% low gelling temperature (LGT) Seaplaque agarose (FMC, USA), kept at about 50°C is mixed at a 1:1 ratio (volume/volume) with the cell suspension. The mixture is incubated for 10 minutes are 50°C.

3. The mixture is immediately dispensed into the 100 µl slots of an appropriate Perspex mold (Bio-Rad Laboratories ltd., Hercules, CA, USA), covered with tape on one side. The mold is put on ice for 5-10 minutes to allow the blocks to solidify. The tape is then gently removed and the solidified blocks pushed out with an appropriate apparatus (Bio-Rad Laboratories ltd., Hercules, CA, USA).

4. Each block is collected in 500 µl of SarE (1% Sarcosyl, 0.5 M EDTA, pH 9.5).

5. Proteinase K (Sigma Chemical Co., St. Louis, MO, USA) is added to a final concentration of 0.5 mg/ml.

6. Following a 30 minute incubation at room temperature, the blocks are incubated at 55°C overnight. (Note that no agitation is required and that longer Proteinase K incubations have proved detrimental to DNA).

7. The blocks are rinsed twice with double-distilled water, and washed three times for two hours and once overnight in 14 volumes of 0.5 TE (10 mM Tris, 0.5 mM EDTA, pH 7.4), by gentle rotation. 0.1 mM PMSF (phenylmethylsulphonylfluoride, Sigma Chemical Co., St. Louis, MO, USA) is added to the first two washes to block protease activity. This is particularly important when restriction digestion is to be performed. The blocks can

be stored at 4°C in 50 mM EDTA (pH 8.0) for several months without detectable DNA degradation.

8. For neutral-neutral 2D gel electrophoresis, the blocks are melted and loaded on the first gel (first dimension) and incubated 30 minutes to equilibrate. (Either the entire block or portion thereof may be used.) The first gel is then run 30 minutes at high voltage (4V/cm). It is then run over night at low voltage (1V/cm). The procedure then continues as described herein above for the second dimension.

This protocol provides the reproducible typical arc patterns as shown in the Figures without a separate DNA isolation step. The protocol allows both qualitative and quantitative analysis. The quantitative analysis is improved in this protocol since the same number of cells is used and it is combined with the internal standardization and normalization utilizing mtDNA, etc. as described in Example 10.

EXAMPLE 10

TELOMERIC SEQUENCES IN spcDNA

spcDNA molecules examined for telomeric sequences in Chinese hamster cells.

Utilizing neutral-neutral 2D gel electrophoresis and a telomere repeat oligonucleotide probe, under stringent hybridization conditions, circular DNA arcs were clearly detected indicating the presence of telomere repeat bearing circular molecules in CHO cells. Previous studies [summarized in Gaubatz, 1990] reported various high-, mid- and low- repetitive sequences in spcDNA, as well as unique ones. However, this is the first time telomeric sequences were detected in extrachromosomal DNA in general, and in spcDNA in particular. These telomeric repeat harboring spcDNA molecules are termed *tel-spcDNA*.

Circular arc identity was confirmed using an "arc c ntrol" blot, as follows: LMW from carcinogen treated C631 cells was separated in the same tank as CHO LMW DNA

(not the same lane). Following blotting and hybridization to an SV40 DNA probe the typical arc pattern was observed (see Figures). Superimposition of the two autoradiograms confirmed arcs' identity. Similar controls for arcs' identity were employed in all 2-D gels analyses in these studies.

This finding of telomeric sequences in spcDNA molecules (tel-spcDNA) in CHO cells was expanded to genetically unstable cells, which had been found to posses unique telomere repeat profile by Fluorescent *In Situ* Hybridization (FISH) analysis: The high-profile CO60 cells ("more" telomeric repeats) and their derived lower-profile C9-3 cells (with "less" telomeric repeats).

LMW DNA from CO60 and C9-3 cells was treated by a double digestion with the restriction enzymes *Bgl* I and *Dpn* II, prior to 2-D gel analysis. The digestion served two purposes: First, as both cell lines contain an integrated SV40 genome, capable of amplification under certain conditions, these contaminating DNA molecules had to be eliminated. This digestion eliminated the SV40 DNA, as confirmed by Southern blot analysis with an SV40 DNA probe. A representative sample from the double digest revealed a typical pattern of fragmented SV40 molecules. Second, such digestion, which involves the frequent 4-cutter enzyme *Dpn* II, which is likely to cut every nucleotide, reduces the total number of circular molecules in this sample, but does not cut inside the telomeric repeat. Thus, the circular molecules detected are more likely to contain long and even exclusive stretches of telomeric repeats, instead of sporadic occurrences. Previous analysis (both FISH and hybridization to oligonucleotide and even random-primed probes) could not distinguish between long and short stretches of telomeric repeats.

Following 2-D gel electrophoresis of LMW DNA double digests from CO60 and C9-3 cells in the same tank, the gel was blotted and membranes were hybridized to a

telomere repeat oligonucleotide probe. In both cases arcs' patterns were confirmed as shown in the Figures. Again, arc no. 1, representing the relaxed circular molecules populations, was clearly detected in both CO60 and C9-3 cells. Thus, tel-spcDNA is not limited to CHO cells, but is a general phenomenon common to at least three Chinese hamster transformed cell lines. Furthermore, pre-treatment digestion of the samples indicates that telomeric sequences probably compose large stretches and even entire molecules, as tel-spcDNA could still be detected after digestion. The combined cutting frequency of these two enzymes is high enough to establish that tel-spcDNA probably contain more than just sporadic telomere-like oligomer repeats.

As CO60 and C9-3 each had a different telomere FISH profile, the tel-spcDNA levels was compared between the two samples. This comparison was facilitated by the fact that both DNA samples were separated on the same gel, blotted and hybridized together. PhosphorImager quantification indicated that the circles' arc intensity in CO60 cells is 3-fold higher than that in the C9-3 samples, suggesting a higher tel-spcDNA level in CO60 cells than in C9-3 cells, in accordance with its higher telomere FISH profile.

This measure was obtained by normalizing according to some independent standards. In 2-D gel analysis three different standards are utilized. First, identical amounts of total DNA are loaded on the gel as determined by O.D. measurements. However, as high molecular weight DNA and low molecular weight RNA contaminants are present in both samples in varying degrees this precaution is insufficient.

Therefore, the linear DNA levels detected by the telomere probe are determined by densitometric analysis by PhosphorImager. Still, low molecular weight linear molecules may be generated during preparation of the DNA samples, by breakage of high molecular weight DNA, which

may occur in different extents in the different samples. This may occur when cells are pre-treated by carcinogen, as carcinogen treatment renders the chromosomal DNA particularly fragile, thereby under-estimating the treated DNA sample.

To overcome this difficulty, a third standard, of mitochondrial DNA (mtDNA) normalization as described herein above is utilized. As the hamster 16Kb mtDNA is purified with the LMW DNA fraction, it serves as a constant parameter for the total amount of DNA that was loaded onto the gel and as a marker for the integrity of the circular DNA in the DNA sample. Furthermore, as mtDNA migrates on the 2D gels in a typical pattern that can be easily distinguished from the linear DNA as well as from the arcs of the circular smaller molecules, its signal can be easily analyzed. Following hybridization of the blot to Chinese hamster specific DNA probe (cloned ATPase and COX 3 genes, Cohen, 1995), the mtDNA unique signal is quantified by PhosphorImager.

mtDNA standards do vary with developmentally distantly related cells or between young and aged cells. This aspect is controlled by the use of an absolute standard of cell number or nuclei number as described in Example 9 herein above.

In this case, when DNA samples were simultaneously prepared from two closely related cell lines, and three different independent normalizing standards were utilized, producing similar results, it can be safely conclude that tel-spcDNA levels were indeed elevated in CO60 compared with C9-3 cells. This finding is in accordance with the telomere FISH profile differences between the two cell lines, and with other characteristics previously described indicating that C9-3 cells may be genetically more stable than CO60 cells. It should be noted that tel-spcDNA was also observed in the established rat cell line A2.

tel-spcDNA induction by carcinogen treatment of CHO cell lines

After establishing spcDNA as a common phenomenon in transformed cell lines as well as tel-spcDNA, it was investigated whether tel-spcDNA may not only be spontaneously present in the intrinsically unstable transformed cells, but can also be further elevated by genomic instability inducing agents, such as carcinogens.

As shown above, total spcDNA levels can be induced by carcinogen treatment in both primary and transformed human and rodent cells. Studies compared tel-spcDNA levels in control and MNNG-treated CHO cells.

LMW DNA was prepared from control and MNNG treated CHO cells 96 hours post treatment, and separated on a 2-D gel in the same tank. Following blotting, the membrane was hybridized first to a hamster Cot-1 DNA probe, then stripped and re-hybridized to an mtDNA probe, and, finally, after stripping, to a telomere repeat oligonucleotide probe. Arcs' identity was determined by an appropriate control, as described above.

Both total spcDNA quantities (as shown above), as well as tel-spcDNA are elevated following carcinogen treatment. PhosphorImager quantification of circular and linear arcs signals as well as mitochondrial DNA signals substantiated this finding, indicating that tel-spcDNA levels were elevated 10-fold by carcinogen treatment. This difference exceeds the total spcDNA elevation detected by carcinogen treatment (3.8-fold). Note, Mitochondrial DNA quantities are reported to remain unchanged in carcinogen treated cells [Sunnerhagen *et al.*, 1989]. Furthermore, when taking into consideration the fact that carcinogen treated cells may be arrested and enlarged (and therefor contain occasionally more mtDNA per cell), spcDNA may actually be underestimated in carcinogen-treated cells relative to normal cells, yet high levels of induction are found.

tel-spcDNA levels are elevated in CHO cells following treatment with the carcinogen MNNG. Furthermore, tel-spcDNA induction exceeds the general induction of total spcDNA by MNNG, as detected by the hamster Cot-1 probe.

In order to investigate whether tel-spcDNA induction by carcinogen treatment is indeed a general phenomenon in Chinese hamster cell lines, 2-D gel analysis was performed on similar amounts (24 μg) of LMW DNA prepared from control and MNNG-treated C9-3 cells. As before, DNA samples were subjected to a double digestion with *Bgl* I and *Dpn* II prior to loading on the gel, to eliminate possible SV40 amplification products, and to reduce the possibility of cross hybridization of the telomere probe with sporadically occurring short stretches of telomere repeats.

Following blotting, membranes were hybridized to a telomere repeat oligonucleotide probe, stripped and re-hybridized to total low molecular weight DNA probe, detecting both mtDNA and total genomic DNA signals. Quantification was performed by PhosphorImager. In contrast to CHO cells, where tel-spcDNA induction exceeded that of total DNA, here total spcDNA is 10-fold induced while tel-spcDNA induction is greatly reduced to only a 2-fold increase in tel-spcDNA levels in treated cells, compared to their non-treated counterparts. The comparison between total-spcDNA induction and tel-spcDNA induction must be interpreted with care as the pre-treatment with the restriction endonucleases clearly has different effects on these two fractions. Note, the mtDNA signals were fuzzy, probably due to mtDNA digestion by the 4-cutter *Dpn* II, as discussed above. Thus, spcDNA signals were normalized according to the linear arc, although similar results were obtained by quantification according to mtDNA standards.

tel-spcDNA is induced in carcinogen treated C9-3 cells, although this induction is less pronounced than

the one detected following MNNG treatment of CHO cells. We hypothesized that this may reflect an intrinsic property in the reaction of C9-3 cells to carcinogen treatment. Note, that another genomic instability phenomena, MNNG-induced SV40 DNA amplification, is also suppressed in C9-3 cells. Thus, while the spontaneous generation of tel-spcDNA is probably a common phenomenon in Chinese hamster transformed cells, carcinogen treatment effects on it may vary, depending on the specific intracellular conditions.

To establish the normal background tel-spcDNA profile in primary cultured Chinese hamster cells, 2-D gel analysis of LMW DNA produced from primary cultured Chinese Hamster Embryo (CHE) cells. tel-spcDNA can be detected in primary cultured Chinese hamster cells at very low levels. However, tel-spcDNA levels in CHE cells are enhanced following MNNG treatment. Quantification of tel-spcDNA from the control and MNNG-treated CHE cells was performed using the mtDNA internal normalizing standard. According to two different normalizing standards the control samples, the tel-spcDNA arc signal in MNNG treated cells was approximately 8-fold higher than the weak signal obtained in control cells, indicating a total ~10-fold increase in spcDNA levels following carcinogen treatment.

tel-spcDNA is rare in primary cultured CHE cells, compared with the readily detected relatively high amounts in transformed cells. However, tel-spcDNA amounts can be significantly enhanced following carcinogen treatment. Enhanced tel-spcDNA levels were also observed in control cells harvested after culturing for prolonged times. A similar effect was noted in total spcDNA in normal human fibroblasts, where the amount of spcDNA increases as the cells reach confluence. These findings are in agreement with previous experiments which indicate that elevated levels of spcDNA were found in confluent cells in comparison with log-phase

counterparts. Similarly, enhanced levels of spcDNA were detected in *in vitro* and *in vivo* senescent cells [Kunisada *et al.*, 1985, Yamagishi *et al.*, 1985], and elevated total spcDNA levels in senescent CHE cells. However, the normalization and quantization protocols as shown herein allow measurement no matter the cellular stage.

tel-spcDNA in human cells

Studies herein above on normal and tumor human tissues revealed that total spcDNA is easily detected in DNA isolated from tissue samples, in contrast with the difficulties encountered with spcDNA detection in human primary cultured cells. For these studies normal colon and colon carcinoma tissue was employed as above.

LMW DNA was extracted by the Hirt procedure [Hirt, 1967] from ground frozen human tissue samples; paired normal colon tissue and colon carcinomas from the same patients. These samples are *in vivo* counterparts of control and treated or primary and immortalized *in vitro* cultured cells.

After the DNA samples were separated on a 2-D gel, blots were hybridized first with a human mtDNA specific probe (known to remain undeleted even in aged mitochondria), the stripped blot was then re-hybridized to a human Cot-1 probe, and finally, following another stripping, to a telomere oligonucleotide probe.

Both total spcDNA and tel-spcDNA arcs were readily detected. Similar arcs were also detected in another pair of normal colon tissue and colon carcinoma from a different patient. Thus, tel-spcDNA is not limited to *in vitro* cultured cells, but can also be detected in tissue samples. Furthermore, tel-spcDNA is detected in the normal colon tissue samples in lower levels, enhancing the impression that it may be a normal phenomenon, which is de-regulated in transformed cells.

tel-spcDNA levels were quantified according to mtDNA standards. While mtDNA quantities are roughly the same

(15% more mtDNA in normal colon than in colon carcinoma), tel-spcDNA is induced 2-fold, as a 100% increase in arc intensity is observed in the tumor, compared to the normal tissue sample. Quantification of another pair of normal colon and colon carcinoma tissue samples from another patient yielded similar results.

The mtDNA probe utilized was previously found to be specifically undeleted during aging processes in various tissues [Lee *et al.*, 1994], thereby minimizing the effect of mtDNA deletions on the quantitative analysis.

In summary, the neutral-neutral 2D gel electrophoresis technique to measure the occurrence of heterogeneous incrementally sized spcDNA and the results presented herein show a clear correlation between the appearance and/or enhancement of spcDNA in various cells and genomic instability. The factors responsible for the transient or stable accumulation of spcDNA are shown to be exogenic, like exposure to environmental agents, genetically programmed, such as aging and differentiation, genetically inherited such as cells from FA patients, or somatically disordered such as in the processes of transformation and tumor progression. In general, it appears that in the analysis of spcDNA set forth in the present invention will serve as a marker for detection and for the determination of genomic instability.

Throughout this application, various publications, are referenced by citation or by number and patents by patent number. Full citations for the publications referenced by number are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology

which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

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CLAIMS

What is claimed is:

1. A method of determining genomic instability associated with heterogeneous incrementally sized circular DNA formation by

isolating intact genomic DNA from a cell sample;

analyzing the DNA by a neutral-neutral buffer pH 2D gel electrophoresis; and

determining the presence an electrophoresis arc pattern as a positive determination of heterogeneous incrementally sized circular DNA molecules whereby genomic instability is indicated.

2. The method of claim 1 further characterized by hybridizing a probe containing a repetitive nucleic acid sequence to the genomic DNA of claim 1 after said electrophoresis.

3. The method of claim 2 wherein the probe is for a highly repetitive nucleic acid sequence.

4. The method of claim 3 wherein the probe is for Cot-1 DNA.

5. The method of claim 2 wherein the repetitive nucleic acid sequence is a telomeric sequence.

6. The method of claim 2 wherein the probe is another cellular sequence.

7. The method of claim 1 wherein said isolating intact genomic DNA from a cell sample is accomplished by

mixing cells from a cell sample with low gelling temperature agarose in a 1:1 ratio;

forming a block of the cells and agarose;

treating the block with proteinase;

melting the block and loading it on the first dimension of the 2D gel and running the gel for 30 minutes at 4V/cm whereby the genomic DNA enters the first dimension of the 2D gel.

8. The method of claim 1 wherein normalization of DNA content of the gels is performed by measuring mitochondrial DNA (mtDNA).

9. A kit for determining genomic instability according to the method of claim 1 including means for isolating a cell sample and isolating genomic DNA from the cell sample, means for performing a neutral-neutral buffer pH 2D gel electrophoresis and means for determining the presence of heterogeneously incrementally sized circular DNA molecules.

10. A method of screening for a possible carcinogen by

establishing a cell culture;

exposing the cell culture to a compound;

isolating genomic DNA from the cell culture;

analyzing the DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of heterogeneously incrementally sized circular DNA molecules from the electrophoresis arc pattern whereby if the circular DNA is present the compound is determined to be a possible carcinogen.

11. The method of claim 10 wherein the cell culture is an chinese hamster SV40 transformed cell culture.

12. The method of claim 10 wherein the cell culture is a normal human fibroblast cell culture.

13. A method of determining a pre-malignancy condition in cells capable of malignancy by

isolating genomic DNA from a cell sample;

analyzing the DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of heterogeneously incrementally sized circular DNA molecules from the electrophoresis arc pattern whereby if the circular DNA is present it is indicative that the cells are determined to be pre-malignant.

14. A method of determining if a subject is susceptible to a carcinogen by

culturing a cell sample from a subject;

exposing the cell sample culture to a carcinogen;

isolating DNA from the cell culture;

analyzing the DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of heterogeneous incrementally sized circular DNA molecules from the electrophoresis arc pattern whereby if the circular DNA is present it is indicative that the subject is susceptible to the carcinogen.

15. A method of determining if a subject has been exposed to a carcinogen capable of inducing heterogeneously incrementally sized circular nuclear DNA molecules by

isolating mitochondrial and nuclear DNA from a cell sample from a subject possibly exposed to a carcinogen;

analyzing the mitochondrial and nuclear DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of an increase in heterogeneous sized circular nuclear DNA molecules from

the electrophoresis arc pattern in comparison to circular mitochondrial DNA molecules whereby if an increase is found it is indicative the subject has been exposed to the carcinogen.

16. The method of claim 15 wherein the cell sample is selected from skin fibroblasts, lymphocytes cells from nasal or buccal smears

17. A method according of determining if a subject may be undergoing premature aging by

isolating genomic DNA from a cell sample from a subject possibly undergoing premature aging;

analyzing the DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of heterogeneous sized circular DNA molecules from the electrophoresis arc pattern whereby if present it is indicative that the subject may be undergoing premature aging.

AMENDED CLAIMS
[received by the International Bureau on 1 October 1997 (01.10.97); new claims 18-24 added; remaining claims unchanged (5 pages)]

the electrophoresis arc pattern in comparison to circular mitochondrial DNA molecules whereby if an increase is found it is indicative the subject has been exposed to the carcinogen.

16. The method of claim 15 wherein the cell sample is selected from skin fibroblasts, lymphocytes cells from nasal or buccal smears

17. A method according of determining if a subject may be undergoing premature aging by

isolating genomic DNA from a cell sample from a subject possibly undergoing premature aging;

analyzing the DNA by a neutral-neutral 2D gel electrophoresis; and

determining the presence of heterogeneous sized circular DNA molecules from the electrophoresis arc pattern whereby if present it is indicative that the subject may be undergoing premature aging.

18. A method of determining genomic instability characterized by the phenotype of heterogeneous incrementally sized circular endogenous genomic DNA formation in a cell sample comprising the steps of:

isolating low molecular weight uncut DNA from a cell sample wherein the low molecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low molecular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis;

determining the electrophoresis arc pattern produced by the hybridization probes wh rein the presence of an

arc pattern showing heterogeneous incrementally sized circular DNA molecul s of endogenous genomic DNA indicates genomic instability.

19. A method of screening for a possible carcinogen characterized by the phenotype of an increase in heterogeneous incrementally sized circular endogenous genomic DNA formation in a cell sample by

establishing a cell culture;

exposing the cell culture to a compound which is a possible carcinogen;

isolating low molecular weight DNA from a cell sample wherein the low molecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low molecular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis; and

determining the electrophoresis arc pattern produced by the hybridization probes wherein if there is an increase in the presence of heterogeneously incrementally sized circular DNA molecules over controls as determined from the electrophoresis arc pattern the compound is determined to be a possible carcinogen.

20. A method of determining a pre-malignancy condition in cells capable of malignancy characterized by the phenotype of an increase in heterogeneous incrementally sized circular endogenous genomic DNA formation in the cells by

isolating low molecular weight DNA from a cell sample of cells with a possible pre-malignancy condition

wherein the low m lecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low molecular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis; and

determining the electrophoresis arc pattern produced by the hybridization probes wherein the presence of an arc pattern indicating an increase of heterogeneous incrementally sized circular DNA molecules of endogenous genomic DNA over normal controls is indicative that the cells are pre-malignant.

21. A method of determining if a subject is susceptible to a carcinogen characterized by the phenotype of an increase in heterogeneous incrementally sized circular endogenous genomic DNA formation in the cells by

culturing a cell sample from a subject;

exposing the cell sample culture to a carcinogen;

isolating low molecular weight uncut DNA from the exposed cell sample wherein the low molecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low molecular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis; and

determining th electr phoresis arc pattern produced by the hybridization probes wherein the presence of an

arc pattern indicating an increase of heterogeneous incr mentally sized circular DNA molecules of endogenous genomic DNA over normal controls is is indicative that the subject is susceptible to the carcinogen.

22. A method of determining if a subject has been exposed to a carcinogen capable of inducing the phenotype of an increase of heterogeneously incrementally sized circular endogenous genomic DNA formation by

isolating low molecular weight DNA from a cell sample of cells from a subject possibly exposed to a carcinogen wherein the low molecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low molecular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis; and

determining the electrophoresis arc pattern produced by the hybridization probes wherein the presence of an arc pattern indicating an increase of heterogeneous incrementally sized circular DNA molecules of endogenous genomic DNA over normal controls is indicative the subject has been exposed to the carcinogen.

23. A method according of determining if a subject may be undergoing premature aging characterized by the phenotype of heterogeneous incrementally sized circular endogenous genomic DNA formation in a cell sample comprising the steps of:

isolating low molecular weight DNA from a cell sample from a patient possibly undergoing premature aging wherein the low molecular weight DNA contains genomic episomal DNA and mitochondrial DNA;

analyzing the low mol cular weight episomal genomic DNA by a neutral-neutral pH 2D gel electrophoresis including normalizing DNA content of the gels using mitochondrial DNA as a reference;

hybridizing with a probe containing an endogenous non-viral genomic repetitive nucleic acid sequence and a probe to a mitochondrial DNA sequence to the low molecular weight DNA after said electrophoresis;

determining the electrophoresis arc pattern produced by the hybridization probes wherein the presence of an arc pattern indicating an increase of heterogeneous incrementally sized circular DNA molecules of endogenous genomic DNA over normal controls is indicative that the subject may be undergoing premature aging.

24. The method of claims 18 to 23 wherein said hybridizing step is further characterized by a first hybridization by one probe, recording the results and removing the first probe and then rehybridzing with a second probe and recording the results.

25. The method of claims 18 to 23 wherein the probe to a mitochondrial DNA sequence is selected to a sequence that is undeleted during aging processes.

26. The method of claims 18 to 23 wherein the episomal low molecular weight DNA is isolated as a Hirt supernatant DNA fraction.

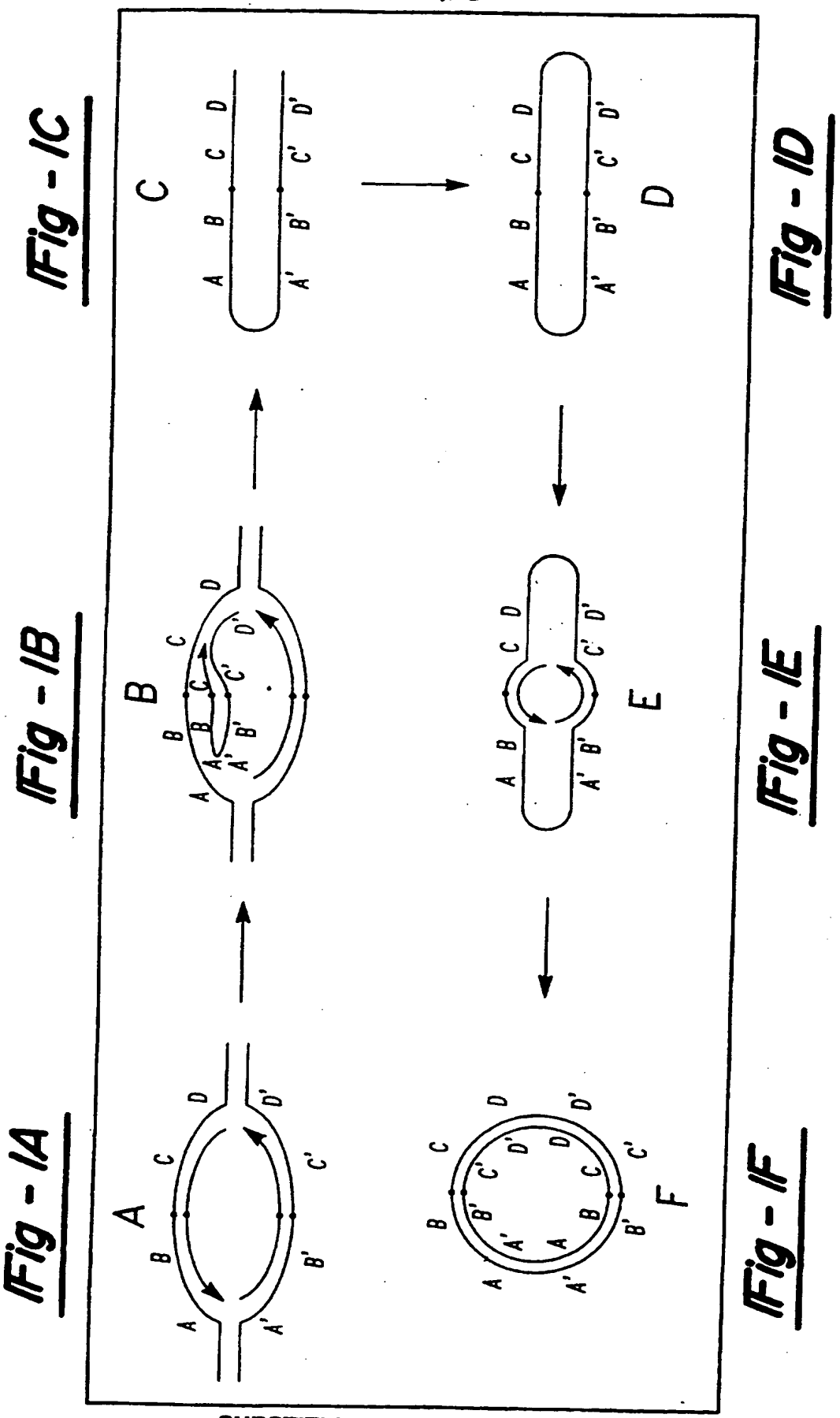


Fig - 2A

Fig - 2B

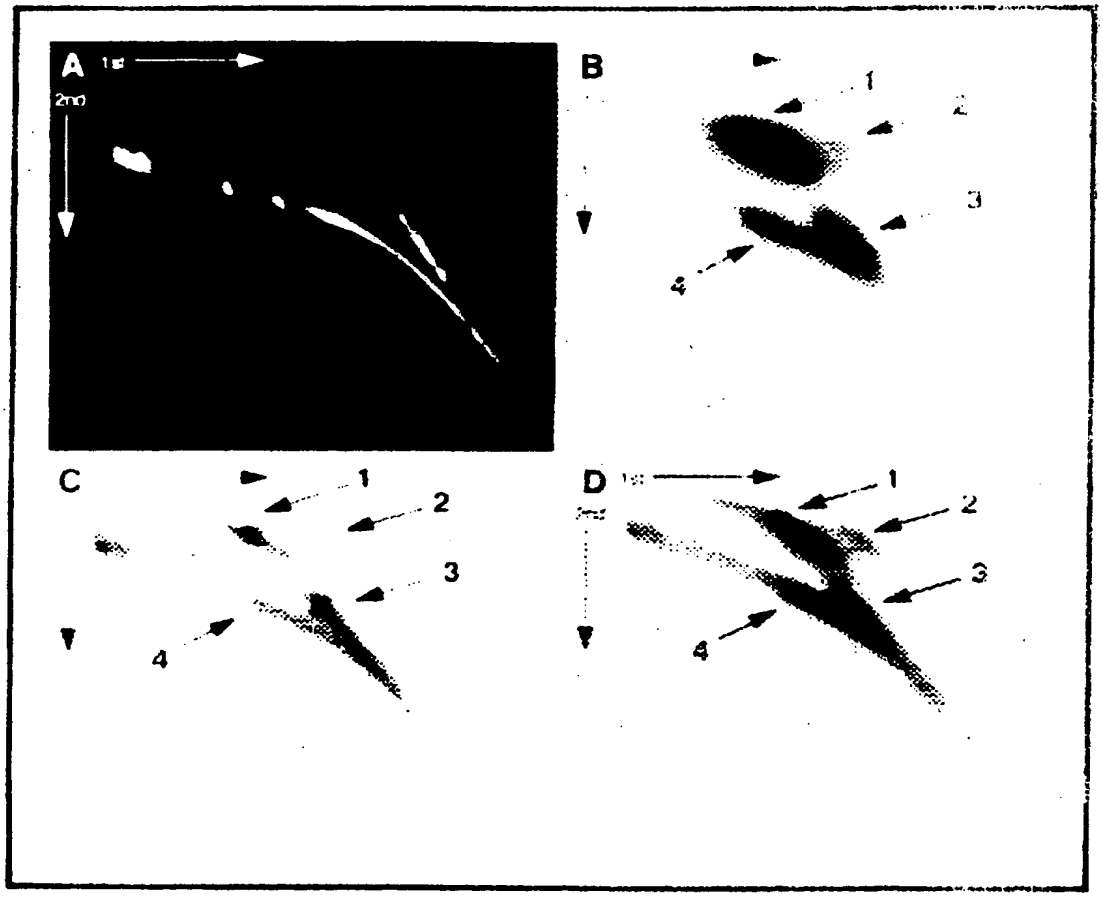


Fig - 2C

Fig - 2D

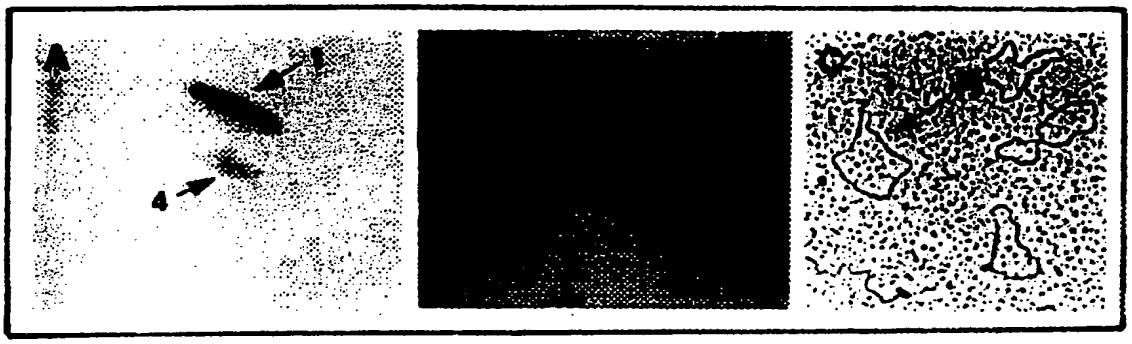


Fig - 3A Fig - 3B Fig - 3C

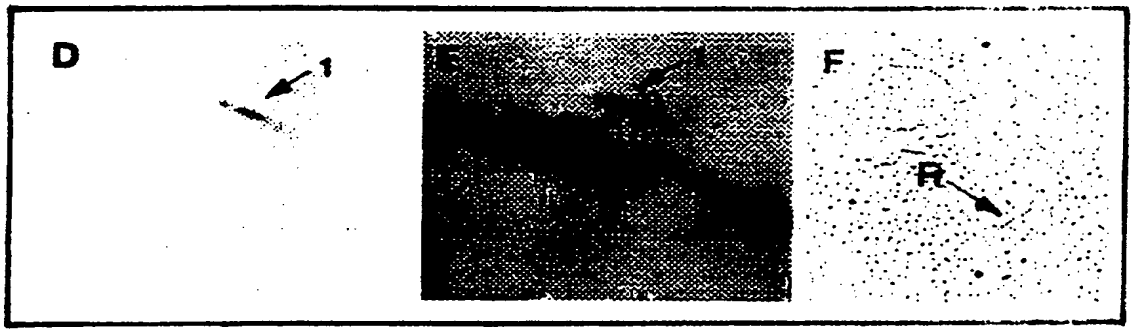


Fig - 3D Fig - 3E Fig - 3F

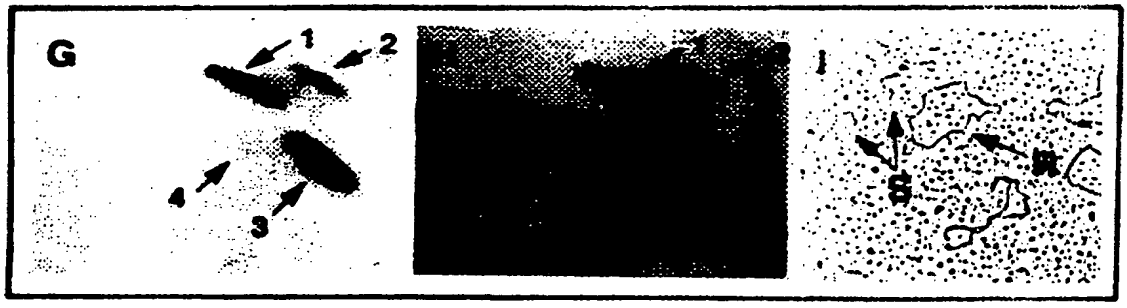


Fig - 3G Fig - 3H Fig - 3I

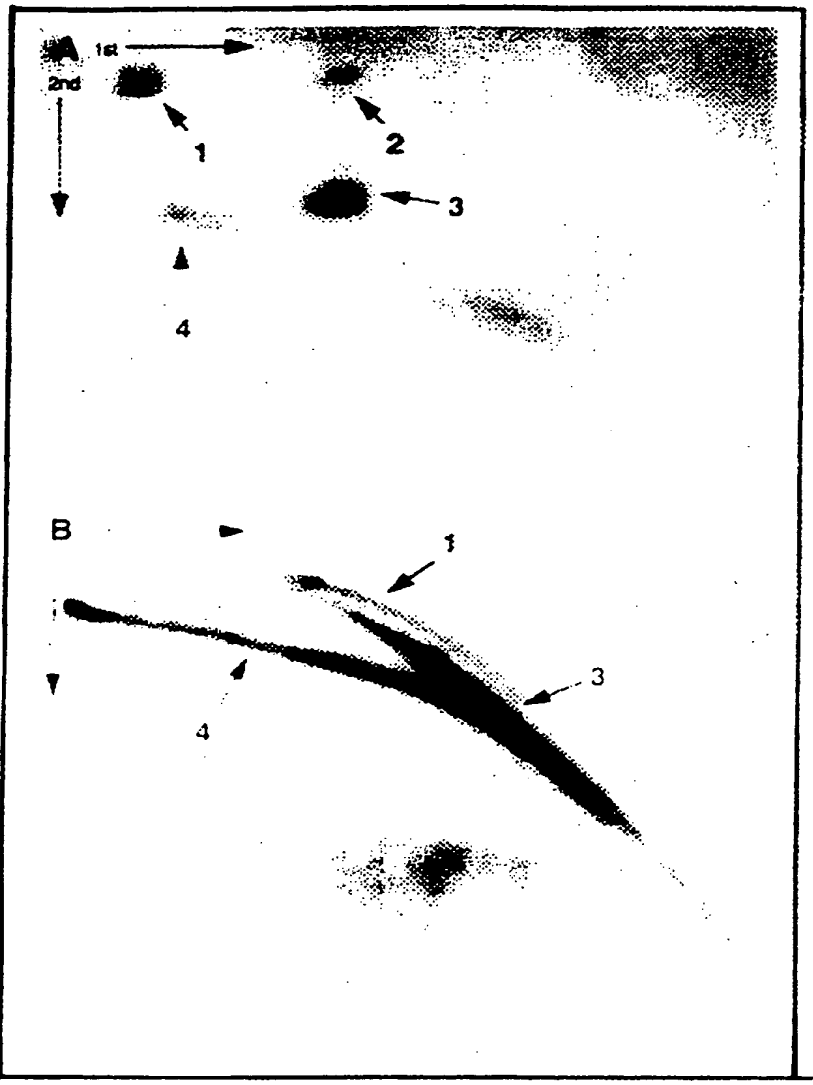


Fig - 4A

Fig - 4B

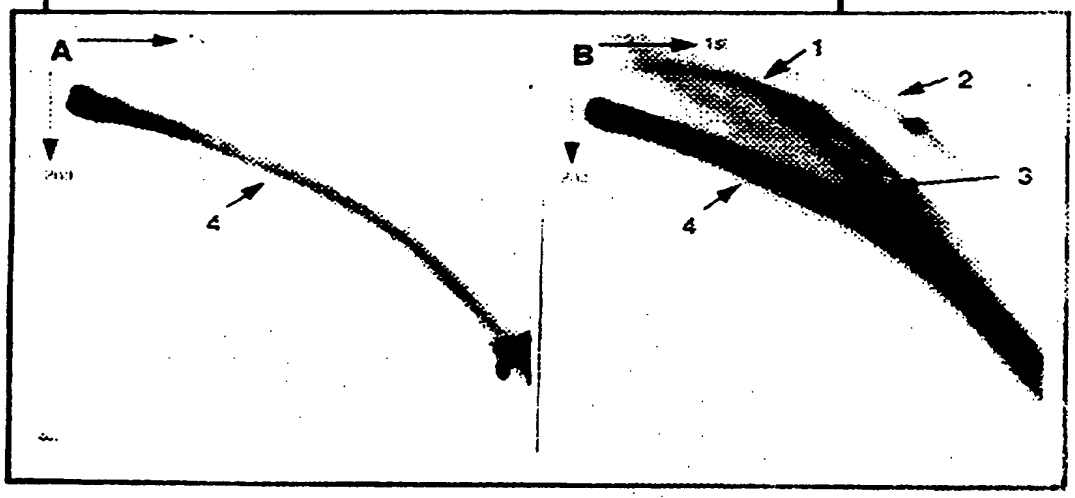


Fig - 5A

Fig - 5B

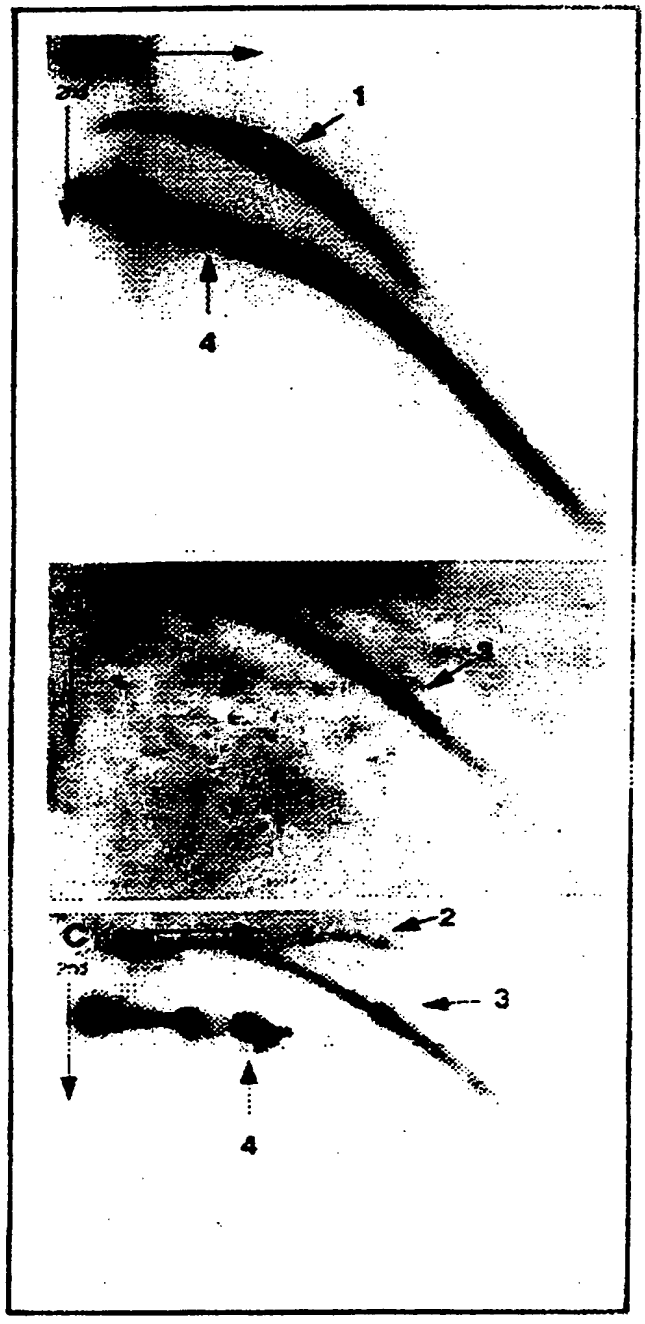


Fig - 6A

Fig - 6B

Fig - 6C

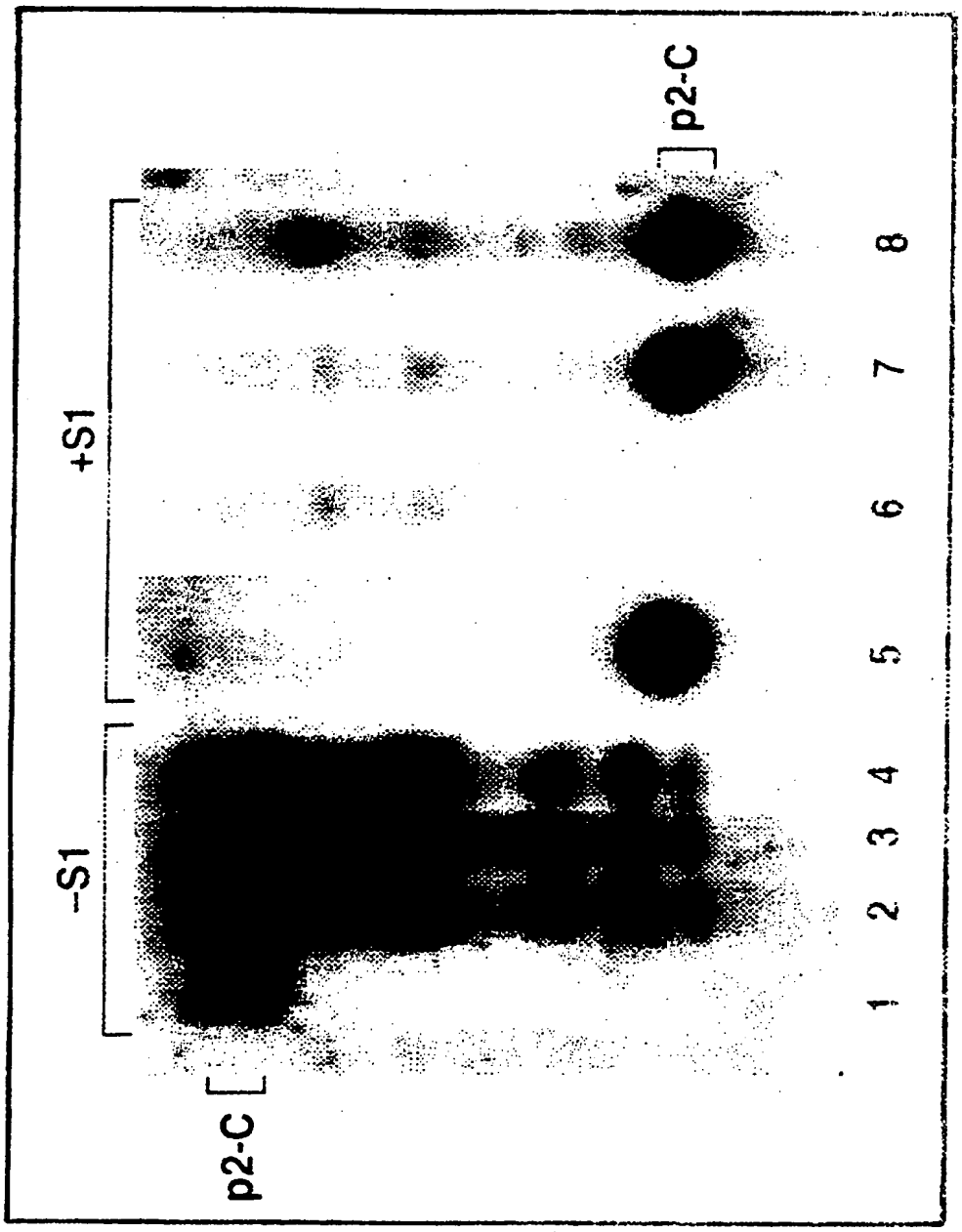


Fig - 7

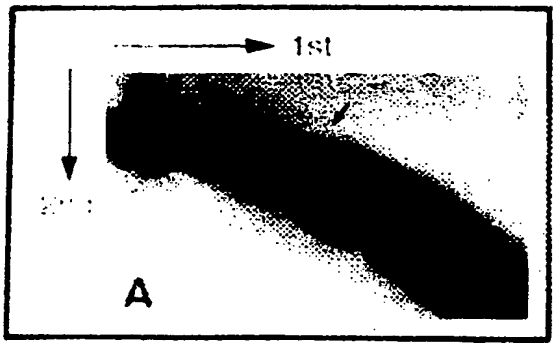


Fig - 8A

Fig - 8A'

Fig - 8B

Fig - 8B'

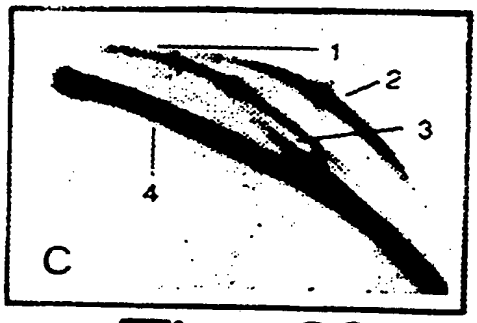
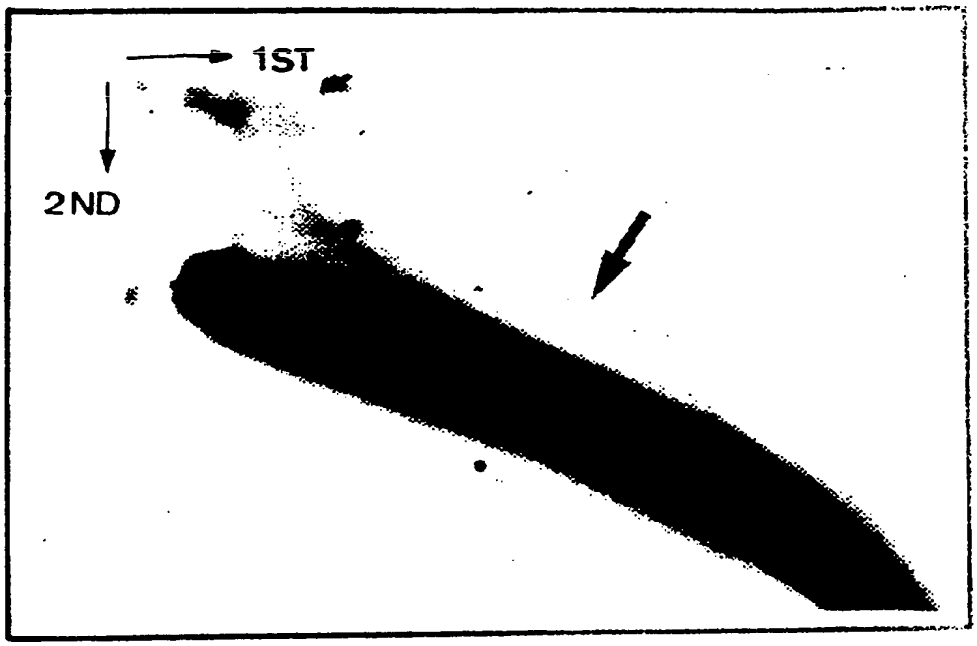
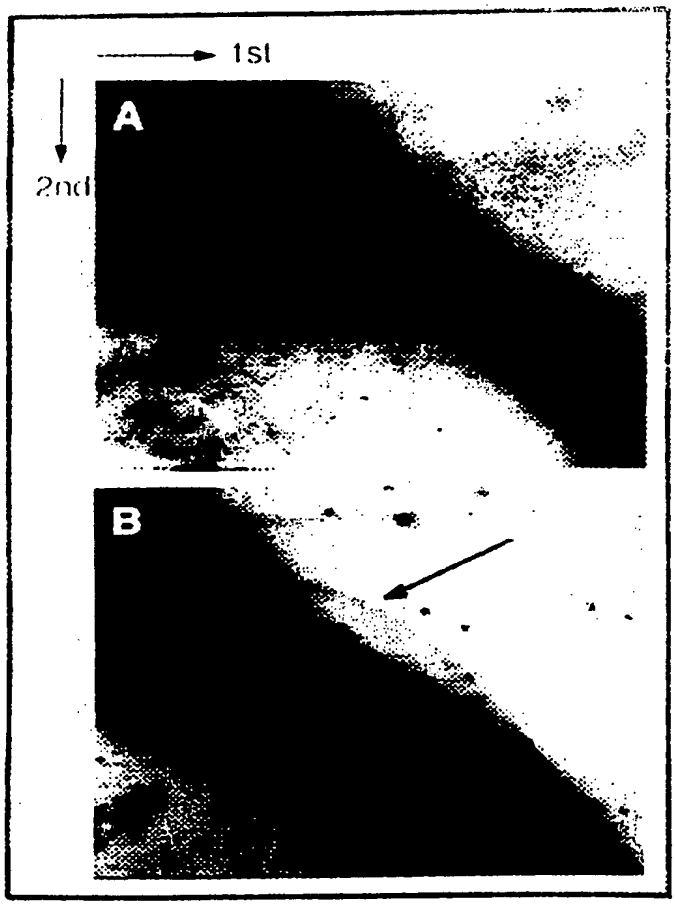


Fig - 8C

Fig - 8D



IFig - 9



IFig - 10A

IFig - 10B

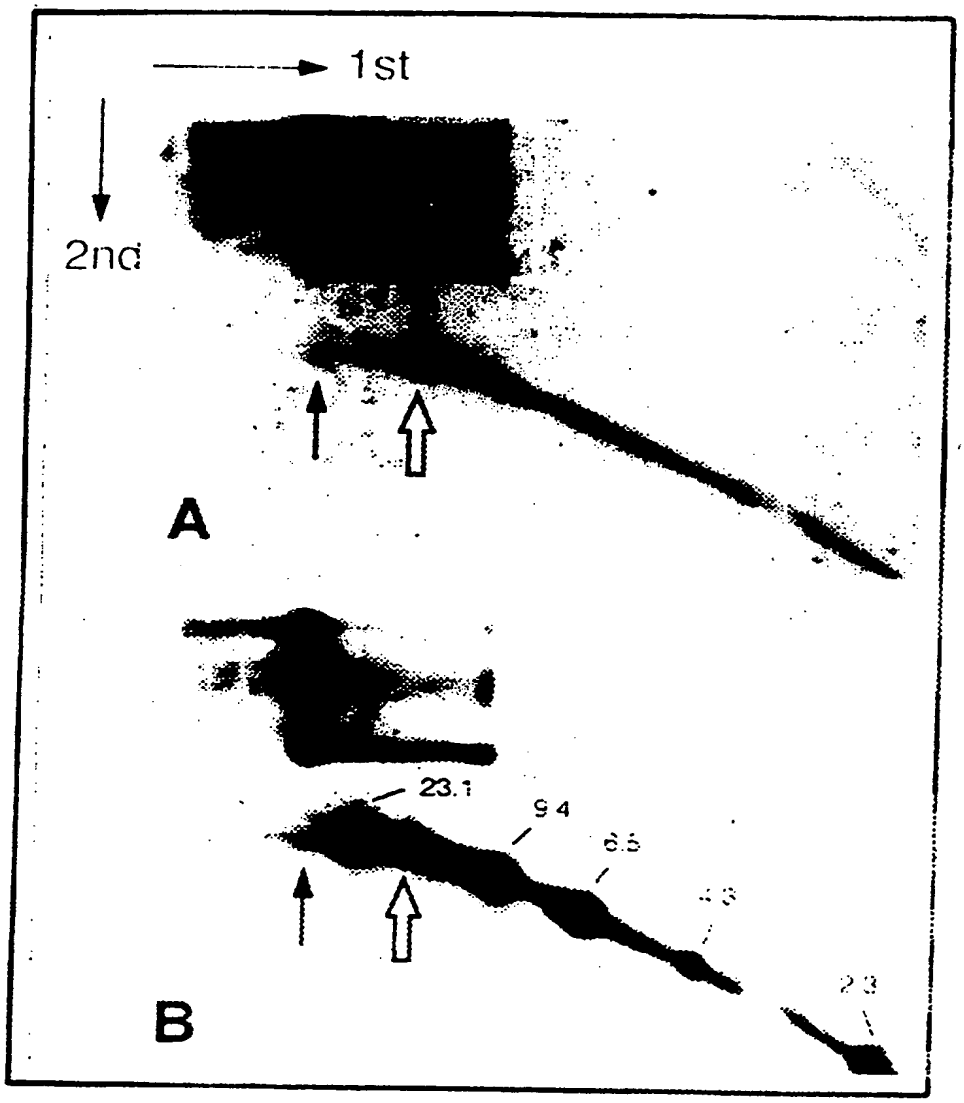


Fig - IIA

Fig - IIB

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06368

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12 Q 1/68; G01N 27/26
US CL :435/6, 810; 204/182.8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 810; 204/182.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SNIJDERS et al. Human Papillomavirus (HPV) type 16 and 33 E6/E7 region transcripts in tonsillar carcinomas can originate from integrated and episomal HPV DNA. J. Gen. Virol., 1992. Vol. 73. pages 2059-2066, especially pages 2060, 2061, 2063-2064.	1-7, 9-16
X	DAS et al. Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 DNA in cervical preneoplastic and neoplastic lesions. J. Gen. Virol. September 1992. Vol. 73. pages 2327-2336, especially the abstract, page 2328, figures 4 and 5	1-7, 9-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
08 JULY 1997

Date of mailing of the international search report
01 AUG 1997

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BNSDOCID: <WO___9739149A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06368

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, CANCERLIT, BIOSIS, MEDLINE, EMBASE, SCISEARCH, LIFESCI, INPADOC
search terms:
2D gel electrophoresis, two dimensional, genomic instability, circular, double minute, cancer, tumor, transform, chromosome, carcinogen, mitochondrial, arc, fibroblasts, lymphocytes, nasal, buccal

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06368

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LITTLE et al. Initiation of Latent DNA replication in the Epstein-Barr virus genome can occur at sites other than the genetically defined origin. Mol. Cell. Biol. May 1995. Vol. 15. pages 2893-2903, especially page 2893, column 1, and figure 3.	1-7, 9-16
X	WELLINGER et al. Origin activation and formatin of single-strand TG1-3 tails occur sequentially in late S phase on a yeast linear plasmid. Mol. Cell. Biol. July 1993. Vol. 13. pages 4057-4065,especially page 4057, column 2, and 4060-4061.	1-3, 5, 9
Y	SCHIMKE et al. Overreplication and recombination of DNA in higher eukaryotes: potential consequences and biological implications. Proc. Natl. Acad. Sci. USA. April 1986. Vol. 83, pages 2157-2161, see entire document.	1, 2, 6, 9-11, 13-17
X --- Y	OPPENHEIM, A. Separation of closed circular DNA from linear DNA by electrophoresis in two dimensions in agarose gels. Nucl. Acids Res., 1981. Vol. 9. pages 6805-6812, see entire document.	9 ------- 1-3, 6, 10, 11, 13-17
Y --- A	GAUBATZ et al. Extrachromosomal circular DNAs and genomic sequence plasticity in eukaryotic cells. Mutation Res., 1990. Vol. 237. pages 271-292, especially the abstract and page 283.	1-3, 10-17 ------ 8